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WALLACE, DONNA GALEAZZI. 2,3,5-Triphenyltetrazolium Chloride: Effect on Neurospora crassa. (1970) Directed by: Dr. James F. Wilson. pp. 80

The tetrazolium salt, 2,3,5-triphenyltetrazolium chloride (TTC), inhibits germination of conidia of all Neurospora crassa strains tested in concentrations exceeding 600 $\mu\text{g/ml}$. At 50 $\mu\text{g/ml}$ there is a dichotomous response to the salt. Some strains are completely inhibited; others, completely resistant. Tetrad analyses of crosses between resistant and sensitive strains provided evidence for the existence of both nuclear and extrachromosomal determinants of the dichotomous response. The nuclear determinants (TTC^R, TTC^S) are alleles of a single gene, closely linked to mating type in linkage group I. The extrachromosomal determinant is associated with the maternally inherited characteristics of [mi-1], and, to a lesser extent, with those of [mi-2] and [mi-4]. In the St. Lawrence-Oak Ridge strains and the Emerson strains mating type A is TTC^R, mating type a is TTC^S. Both mating types of the Rockefeller-Lindegren strains are TTC^R. An [mi-1] strain with a TTC^R nuclear allele is more resistant than an [mi-1] strain with a TTC^S allele, suggesting a different mechanism for nuclear and cytoplasmic-based resistance.

Experimental results show that the TTC effect is inhibitory, not lethal, and is restricted to the ungerminated conidium. Conidia incubated in the presence of TTC before plating on TTC-free medium were not inhibited, indicating the effect was reversible. Sorbose present in the incubation medium caused a slight increase in inhibition.

A concentration of TTC inhibitory for mating type a induced abnormal vacuolation in a conidia but had no effect on A. In the presence of TTC, red crystals could be observed in vegetative hyphae of mating type a at least one hour before they were visible in A, but both mating types showed identical response to microinjected TTC. This difference in effect of externally and internally applied TTC suggests that the response of the Neurospora mating types is based on a variation in membrane permeability, rather than on metabolism. The mitochondrial mutant [mi-1] is resistant to TTC even when it has a TTC^S nuclear allele, and the [mi-1] phenotype eventually replaces that of wild type when a mixture of the two types of mitochondria are present in a common cytoplasm. Such a mixture (heterochondrion) can be produced by microinjection of [mi-1] mitochondria into wild type. It was then possible to follow the change in mitochondrial populations in the heterochondrion by plating conidia on medium containing TTC.

2,3,5-TRIPHENYLTETRAZOLIUM CHLORIDE:

EFFECT ON NEUROSPORA CRASSA

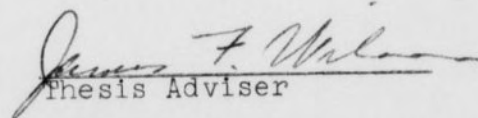
by

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INTRODUCTION

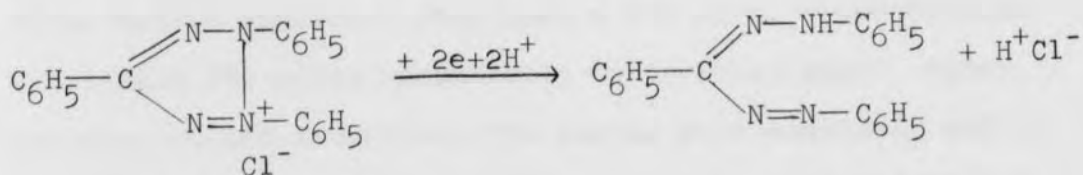
The tetrazolium salts are a group of closely-related compounds first described late in the nineteenth century. Biologists became interested in them in the 1940's when it was found that the salts are reduced by living cells. They are used in a variety of areas of research which, for the most part, utilize their reduction to colored compounds by actively metabolizing cells. They, therefore, can provide a simple visual test for viability under prescribed conditions.

The sciences of agriculture, biochemistry, and microbiology have made much use of the monotetrazolium salt, 2,3,5-triphenyltetrazolium chloride, the first of the tetrazolium salts prepared. Because it had limitations other salts were synthesized, such as neotetrazolium chloride, blue tetrazolium and the iododerivatives. However, since triphenyltetrazolium chloride, or TTC, was prepared first, most of the early applications involved it rather than the others.

Cottrell (1947) and others were able to use TTC to test germinability of seeds; the results showed good agreement with standard germination tests. The seeds tested were wheat, oats, barley, peas, and vetches. The seeds were soaked in tap water overnight, then cut longitudinally to bisect the embryo. One half of each seed was then placed in a petri dish and covered with a 1% TTC solution for 4 hours at 20 C. Within

24 hours results were obtained with TTC while the standard germination test required 10 days or more. Since each seed must be examined, the TTC test is more time-consuming for the tester but does offer a quick evaluation of seed viability.

Mattson, Jenson, and Dutcher (1947) confirmed previous observations of the reaction of TTC with yeast. In addition they stained other viable materials with neutral solutions of TTC; among these were fleshy portions of apples and oranges, gill area of mushrooms, carrot roots, potatoes, and bull spermatozoa. Because it is one of the comparatively few organic compounds which is colored in the reduced state it has a distinct advantage over others. The reaction:



TTC (soluble in
water and colorless)

Formazan (insoluble in
water and red)

This colored, insoluble, reduced form naturally led to use of the tetrazolium salts as oxidation-reduction indicators in biochemical studies such as that carried out by Seligman and Rutenberg (1951) on dehydrogenases. In this type of investigation, the tetrazolium salts offer two advantages over the usual methylene blue reaction in that (1) they are colored on reduction while methylene blue decolorizes and (2) the formazans are not readily reoxidized in air, in contrast to methylene blue. TTC had been

previously used as a substitute for methylene blue by Kun and Abood (1949) in the determination of succinic dehydrogenase. Using TTC in the study of tissue homogenates, they could follow the enzyme activity quantitatively by colorimetric measurements. In 1957, Ling, Su, and Tung showed that the methylene blue system was not completely analogous to the TTC system. The reduction of TTC did not require the same factor or factors as the reduction of methylene blue. Cytochrome c₁ or e was suggested as the factor for TTC in the succinoxidase system.

In 1950 Huddleson and Baltzer were already using TTC in culture medium to differentiate bacterial species and variations within species. They used a 100 µg/ml concentration of sterile TTC added aseptically to tryptose agar. After inoculation and incubation the plates were examined, and it was reported that distinct differences in color and shades of color were observed in the central circular region as well as in the border area of the colonies. In general, the central region showed various shades of red, while the borders displayed delicate pastel tints of green, blue, yellow, and red. Colonial growth phases of *Salmonella* were discernable by various color schemes displayed after incubation on media containing TTC. Other microorganisms examined were *Brucella*, *Shigella*, *Escherichia*, *Alcaligenes*, *Staphylococcus*, and *Streptococcus*. Huddleson and Baltzer recommended a concentration of 25 µg/ml TTC for *Staphylococcus* and certain

members of the genus *Bacillus* because any higher concentrations inhibited growth of these organisms. In 1952 Kennedy and Barbaro reported inhibition of some strains of *Micrococcus* also at a final TTC concentration of 25 $\mu\text{g/ml}$.

In addition to the biological and biochemical applications of the tetrazolium salts, others employing them as chemical reagents have been developed. In 1947 Mattson, et al. found that in an alkaline medium, TTC is reduced by reducing sugars. Mattson and Jensen (1950) developed a technique using TTC to measure colorimetrically the quantity of reducing sugars in a sample. The quantity of formazan produced was proportional to the quantity of reducing sugar in the sample.

Cheronis and Stein (1956) developed a test to detect the reducing functions of organic compounds by using TTC. This test was effective even if the compounds were present in concentrations of a few micrograms per milliliter.

Most of the recent work has had the objective of determining the mode and site of action of the tetrazolium salts. It has been established that they interact with the electron transport system located in the mitochondria of the cell. The tetrazolium salts compete with various components of the electron transport system for hydrogen. Work has been done both on plant and animal mitochondria to determine the point in the electron chain where the tetrazolium salts are reduced. Kalina and Palmer (1968) experimented with mitochondria

from Jerusalem artichoke tubers, while Slater, Sawyer, and Sträuli (1963) worked with mitochondria isolated from rat liver. In general there is agreement on reduction sites for most of the salts tested, regardless of the source of the mitochondria. One observed difference is that in plant studies thiazolyl blue (MTT) and iodonitrotetrazolium chloride (INT) are reduced in the first half of the chain before the antimycin A-sensitive point, while in the animal studies this reduction occurred in the last half.

The nitro-mono- and ditetrazolium salts accept electrons at the initial stages of electron transport, while TTC and tetrazolium blue (BT) accept them at the terminal portion of the chain. Slater, et al. (1963) found that one of the tetrazolium salts, MTT, appears to possess two major reduction sites. INT and, under anaerobic conditions, neotetrazolium chloride (NT) show major as well as minor sites. In general, sites for the reduction of the various tetrazolium salts were determined through the use of inhibitors which would selectively interfere with electron transport at various known sites, i.e., cyanide at cytochromes a/a₃. The inhibition of the reduction of each salt to its formazan after these treatments was used to identify the reduction sites (Nachlas, Margulies, and Seligman, 1960; Slater, et al., 1963; Kalina and Palmer, 1968). The particular salt used and observed here was TTC, a mono-tetrazolium salt also known as red tetrazolium. It is colorless, being reduced to a deep red,

insoluble, triphenylformazan. There appears to be agreement on the cytochrome oxidase region as the reduction site in the electron transport chain (Kalina and Palmer, 1968; Nachlas, et al., 1960; Slater, et al., 1963).

Clark, Greenbaum, and Slater (1965) noted that the reaction sites of neotetrazolium chloride were similar in sequential position to the three proposed sites of oxidative phosphorylation. Using succinate and β -hydroxy-butyrate as substrates, they found that the tetrazolium salts uncoupled oxidative phosphorylation at low concentrations. The decreasing order of effectiveness as uncoupling agents when testing rat-liver mitochondrial preparations was: NT, INT, NBT, TTC, and MTT. Even MTT uncoupled completely at a final concentration of 130 μ M.

Palmer and Kalina (1968) found that tetrazolium can completely uncouple phosphorylation from oxidation in plant mitochondria as well. They found a strong correlation between molecular structure and the ability to uncouple oxidative phosphorylation. The monotetrazolium salts TTC, INT, and MTT all cause complete uncoupling around 100 μ moles/liter, while the di-tetrazolium salts NT, BT, NBT, and TNBT all cause complete uncoupling around 20 μ moles/liter. This work was done using succinate and malate as substrates.

Several of the salts were reduced rapidly with the succinate substrate but not with a malate substrate. Since neither TTC nor NT can accept electrons from the cytochrome

oxidase region of the chain common to both dehydrogenase systems, Palmer and Kalina reasoned that the salts inhibit electron flow between malate and cytochrome b. They did find that the tetrazolium salts can inhibit electron flow from NAD^+ linked substrates to cytochrome. Sato and Sato (1965) and Clark et al. (1965) had shown this to be true in animal tissue.

Kalina and Palmer in 1968 investigated factors affecting the rate of reduction of the tetrazolium salts. They found a definite lag phase in the rate of reduction using a succinate substrate. By adding ATP they completely abolished this lag phase and enhanced the linear rate of reduction. They have two possible interpretations. One is that the ATP may activate a system to transfer electrons to the tetrazolium salts. Lester and Smith (1961) had found no direct transfer of electrons between the chain and the tetrazolium salts. The second is that the ATP affects the penetration into the sites of reduction in the mitochondria. The uptake of the tetrazolium salts into the mitochondria may be both active and passive. The passive diffusion and the energy-requiring active penetration could proceed simultaneously. This would explain why ATP has a greater stimulating effect at low concentrations of tetrazolium salt and why ATP stimulation effect is inhibited by cyanide and azide.

Since the tetrazolium salts are so efficient at disrupting the electron transport chain and uncoupling oxidative phosphorylation, one would think their ability to

inhibit microorganisms would have been thoroughly investigated. A number of authors have made mention of such inhibition, but extensive research specifically addressed to this problem is scarce. Weinberg (1953) reported inhibition of bacteria, actinomycetes, and molds by triphenyl tetrazolium chloride. The molds, which were not specifically identified, tolerated 2500 $\mu\text{g/ml}$ of TTC. In 1958 Brock stated that Weinberg had found that "the filamentous fungi neither reduce tetrazolium nor are they inhibited by it." Interestingly enough, Weinberg's paper contains no such statement.

The results reported in this thesis, using the filamentous fungus, Neurospora crassa, as the test organism, show that this fungus definitely reduces TTC, can tolerate only a small fraction of the concentration used by Weinberg, and what resistance it does have is genetically controlled by both nuclear and cytoplasmic genes.

MATERIALS AND METHODS

Some strains used in the studies are listed in Table 1. The source referred to as FGSC is the Fungal Genetics Stock Center; Department of Biological Sciences; Dartmouth College; Hanover, N.H.; U.S.A. All cultures whose designations are prefixed by NC were isolated from crosses made at the University of North Carolina at Greensboro. Crosses were routinely made on a modified synthetic crossing medium of Westergaard and Mitchell (1947).

Sorbose minimal medium of deSerres, Kølmark, and Brockman (1962) was used for all sorbose platings. A suspension of conidia was made in sterile distilled water and filtered through sterile glass wool in a thistle tube. Conidial counts were done using a hemocytometer chamber. After appropriate serial dilutions, no more than 0.3 ml of inoculum was added either to the liquid medium before pouring into a sterile petri dish (pour-plate technique) or to the surface of the solid medium and distributed by smearing with a triangular glass tool (smear technique). Since an inoculum of more than 0.3 ml was large enough to dilute the TTC present in the medium and cause erroneous results, suspensions were made heavy enough to avoid this situation.

The TTC is reduced chemically if autoclaved with agar so stock solutions were prepared and added to the sterile media

TABLE 1 SOME STRAINS USED IN TTC STUDIES

Culture			Source	FGSC #	Designation
74-OR8-1	<u>a</u>	wt	D.D. Perkins	988	OR-SL <u>a</u> <u>TTC</u> ^s
74-OR23-1	<u>A</u>	wt	D.D. Perkins	986	OR-SL <u>A</u> <u>TTC</u> ^r
ST 4	<u>A</u>	wt	D.D. Perkins	262	ST <u>A</u> <u>TTC</u> ^r
Emerson	<u>A</u>	wt	F.G.S.C.	691	E <u>A</u> <u>TTC</u> ^r
Emerson	<u>a</u>	wt	F.G.S.C.	692	E <u>a</u> <u>TTC</u> ^s
SL 3	<u>a</u>	[<u>mi-2</u>]	F.G.S.C.	1581	SL [<u>mi-2</u>] <u>a</u> <u>TTC</u> ^s
SL 3	<u>a</u>	[<u>mi-4</u>]	F.G.S.C.	1585	SL [<u>mi-4</u>] <u>a</u> <u>TTC</u> ^s
SL 3 (1-8)	<u>a</u>	[<u>mi-1</u>]	F.G.S.C.	1578	SL [<u>mi-1</u>] <u>a</u> <u>TTC</u> ^s
NC-5 117	<u>a</u>	wt	J.F. Wilson		OR-SL <u>a</u> <u>TTC</u> ^r
NC-OR (2-3)	<u>A</u>	wt	J.F. Wilson		NC-OR <u>A</u> <u>TTC</u> ^r
NC-OR (2-7)	<u>a</u>	wt	J.F. Wilson		NC-OR <u>a</u> <u>TTC</u> ^s
NC-37401-NS-10 (2-6)	<u>a</u>	<u>inos</u> , [<u>mi-1?</u>]	J.F. Wilson		NC-RL [<u>mi-1?</u>] <u>a</u>
NC-15300-f ₃ #20	<u>a</u>	<u>pan-1</u> , <u>al-2</u>	J.F. Wilson		NC-RL #20 <u>a</u> <u>TTC</u> ^r or NC-RL <u>a</u>
37401 (11-7)	<u>a</u>	<u>inos</u>	J.F. Wilson		37401 <u>inos</u> <u>a</u>

just prior to pouring plates (Ogur, St. John, and Nagai, 1957). All of the other tetrazolium salts were handled similarly.

The tetrazolium salts used and their sources were:

TTC	triphenyl tetrazolium chloride, Sigma Chemical Co.
NBT	nitro blue tetrazolium chloride, Sigma Chemical Co.
INT	iodonitro tetrazolium violet, Sigma Chemical Co.
NT	neotetrazolium chloride, Nutritional Biochemicals Corp.

The concentration of NBT, INT, and NT used was determined on the basis of the effect of TTC in previous experiments and the fact that by weight their electron-acceptance potentials are about equal (Nachlas, et al., 1960).

On all platings, colonies were counted at 24, 48, 72, and 96 hours or as otherwise noted. Colonies were counted on a Luminescent Colony Counter (New Brunswick Scientific Co., Inc.).

The TTC resistance or sensitivity of a particular strain can be determined by a sorbose plating. However, this technique is too time-consuming and unwieldy for assaying a large number of ascospore isolates. A quick, simple, visual test was devised to analyze a large number of isolates. This spot test consisted of a sorbose agar plate (with a supplement if required) divided into ten pie-shaped wedges by lines drawn on the bottom of a sterile plastic petri dish with a felt tip marker. Each wedge was inoculated with a moistened loop of conidia from the sample. A sterile disc (Whatman 3MM), impregnated with a stock solution (10 mg/ml) of TTC, was placed near the outer edge of the sector. The TTC permeated the

medium in the area nearby. After incubation at 30 C for 24 hours, a zone of inhibition could be observed around the disc in the sectors inoculated with TTC-sensitive strains. TTC-resistant strains showed no zone of inhibition.

Sex tests were routinely done on Corn Meal Agar (Difco No. 0386-01) with 0.2% glucose added. A set of plates was inoculated in the center with a known tester strain, one A, one a. After 7 days, when sufficient protoperithecia were present, the bottoms of the plates were marked with circles. In each circle was smeared a wet loopful of conidia from a strain of unknown mating type. Each isolate was tested on both mating types. After 48-72 hours, the plates could be scored. The unknown formed black perithecia with the opposite mating type only.

The compatibility tests observed microscopically were done by the method of Wilson and Garnjobst (1966). The only modification in the technique was filling the chamber with 15% sucrose-Vogel's (Vogel, 1956) minimal medium rather than 15% sucrose only. This change was made to promote growth and, hopefully, fusion between the two strains once they were on the chamber.

EXPERIMENTAL

Overlay Experiments

Ogur, St. John, and Nagai (1957) reported the successful differentiation of wild type and petite forms of yeast using TTC. Wild type yeast strains contain cytochrome c, b, and a + a₃, but the petite strains contain only c and that in great excess. This difference in cytochrome content is inherited cytoplasmically in yeast, indicating that genetic control of these components of the electron transport chain resides in the cytoplasm.

Ogur, et al. (1957) used agar containing 1 mg/ml TTC with pH adjusted to 7.0 as an overlay on plates containing 3-4 day old colonies of yeast. After 3 hours the plates could be scored. Wild-type colonies reduced the TTC to formazan and turned red. Petite colonies were still white at this time, although they became pink within 24 hours.

The [mi-1] strain of Neurospora crassa has a cytochrome spectrum very similar to that of the petite strains of yeast, and the character is also inherited through the cytoplasm. Since we were searching for a method for differentiation of wild-type and [mi-1] colonies on sorbose plates, we decided to try the TTC overlay technique.

Yeast will grow very well on a neutral medium, but

Neurospora will not. TTC apparently is not reduced (Mattson, et al., 1947) efficiently at higher hydrogen ion concentrations. The method of Ogur, et al. (1957) was modified by adding the TTC without agar with enough Na_2HPO_4 to raise the pH from the original 5.8 which is optimum for the growth of Neurospora, to pH 7.0 at which the TTC should be reduced.

The final concentration of the TTC was 1 mg/ml. After incubation at 30 C for 3 hours, no color developed; however, if allowed to incubated overnight, most of the wild-type colonies did develop red centers. The [mi-1] colonies similarly treated showed no change. If the colonies were allowed to conidiate, the overlay did not work. Therefore, smear plates yielded the best results, since all colonies were on the surface at the same time and in the same growth phase. Unfortunately, some wild-type colonies did not develop color even on the smear plates, so the overlay technique did not give conclusive results.

Inhibition Experiments

TTC was then incorporated into the growth medium in the hope that, even at an unfavorable hydrogen ion concentration (for TTC reduction), longer exposure to the TTC would produce a higher incidence of colored wild-type colonies. This attempt to enhance TTC reduction led to the discovery that wild-type colonies of a mating type failed to appear in the presence of 100 $\mu\text{g/ml}$ TTC, although [mi-1] colonies developed

as usual. This accidental discovery eventually gave us a means of differentiating between wild type and [mi-1] that was actually more versatile, easier to employ, and more accurate than the overlay technique. It eliminated the problems of adding the TTC at exactly the right time and of overlaying all colonies. Some colonies appeared to have an anti-wetting layer over them and as a result required much shaking of the dishes to accomplish the overlay. Incorporation of TTC into the medium also eliminated the problem of adjusting the pH.

Response of Various Neurospora Strains to TTC

The Emerson a FGSC 692 strain used in the inhibition experiments showed evidence in crosses of carrying genes for slow growth. We, therefore, felt it wise to test the response of several other strains to TTC in order to learn whether the behavior of Emerson a was typical or exceptional.

Table 2 gives the other strains tested by sorbose plating of conidia and the response of these strains in terms of per cent inhibition as compared to control plates without TTC. The results indicate that Emerson a is not exceptional in its sensitivity to TTC; the a strains of Oak Ridge-St. Lawrence background respond in the same way. It should also be noted that the response to TTC shows a suspiciously high degree of correlation with mating type. In the Oak Ridge-St. Lawrence strains and the Emerson strains, all the A mating types are

TABLE 2
VARIATION IN STRAIN RESPONSE TO TTC

Strain	Mating Type	% Inhibition
Emerson 5256	<u>A</u>	0
Emerson 5297	<u>a</u>	84
Emerson	<u>A</u>	7
Emerson	<u>a</u>	98
74-OR23-1	<u>A</u>	0
ST 4	<u>A</u>	0
74-OR8-1	<u>a</u>	100
NC-RL-f ₂	<u>A</u>	11
NC-RL-f ₂	<u>a</u>	67
NC-15300-f ₂ <u>pan-1</u> , <u>al-2</u>	<u>a</u>	2
37401 <u>inos</u>	<u>a</u>	0

Plating medium was sorbose minimal agar; inoculated with approximately 100 conidia using the pour-plate technique. Incubated 72 hours at 30 C. Per cent inhibition based on the number of colonies that appeared on control plates containing no TTC. All strains except those designated pan-1, al-2, and inos are wild types. TTC concentrations--100 µg/ml.

resistant to TTC; the a mating types are sensitive. This pattern suggests that the difference in mating type response could be controlled by a nuclear gene linked to mating type and that the mating types have different alleles. In the Rockefeller-Lindegren strains, however, both mating types are TTC-resistant. The NC-RL-f₂ wild type a showed a degree of resistance intermediate to that of OR-SL A and a strains, but since two different RL a nutritional mutants exhibited a very high degree of resistance, it seems probable that the wild type NC-RL a partial sensitivity is an individual peculiarity. The fact that this NC-RL wild type is at least two crosses removed from the wild-type strains from which the mutants were derived strengthens this probability.

Determination of Optimum Plating Conditions

Having established that TTC-sensitivity was not peculiar to Emerson a, experiments were then designed in which the concentration of the conidial inoculum and of the TTC were systematically varied in order to determine optimum plating conditions.

The results of these tests indicated the upper and lower limits of effective concentrations of TTC for the above strains. Concentrations of 50-100 µg/ml served to differentiate between TTC-resistant and TTC-sensitive strains. Table 3 shows the response of Oak Ridge-St. Lawrence a and A wild types to a range of TTC concentrations.

TABLE 3
EFFECT OF TTC CONCENTRATION ON WILD-TYPE CONIDIA

Concentration TTC ($\mu\text{g/ml}$)	OR-SL <u>a</u>	% Inhibition ST <u>A</u>
600	100	98
400	100	96
300	100	69
200	100	36
150	100	14
100	100	0
50	100	2
40	86	-
20	6	-

Platings were done in triplicate on sorbose minimal agar, using 100 conidia per plate. These were incubated for 72 hours at 30 C. Per cent inhibition based on the number of colonies that appeared on control plates containing no TTC.

The effective range of TTC concentrations on Oak Ridge-St. Lawrence a wild type was from 20 $\mu\text{g/ml}$, which produced only 6% inhibition, to 50 $\mu\text{g/ml}$, which inhibited completely. The range for mating type A was 100 $\mu\text{g/ml}$ to 600 $\mu\text{g/ml}$. These data indicate that mating type A can withstand approximately twelve times as much TTC as mating type a.

All of the [mi-1] strains tested could tolerate 50-100 $\mu\text{g/ml}$ TTC regardless of their background. This made the TTC system a feasible technique for the differentiation of [mi-1] and wild type provided a mating types were used.

Emerson a was sorbose plated at 4 conidial concentrations in order to determine limits if there indeed were any. At 100 $\mu\text{g/ml}$ as many as 10,000 conidia were inhibited; at 50 $\mu\text{g/ml}$ more than 100 conidia would allow some survival. The figures in Table 4 show less inhibition at 50 $\mu\text{g/ml}$ TTC and 100 conidia than Table 3 because the strain was different, and the plating technique was slightly different. The results in Table 4 were obtained by using the smear plate technique while the results in Table 3 were obtained by using the pour plate technique. In subsequent experiments using pour plates, 50 $\mu\text{g/ml}$ TTC, and 100 conidia, we attained 100% inhibition.

Genetic Analyses

The characterization of the determinants of the TTC response required over 60 crosses from which at least 200 ordered asci were isolated and analyzed. It was found that

TABLE 4
EFFECT OF CONIDIAL CONCENTRATION ON TTC RESPONSE

Number of Conidia	Number of Colonies	
	100 μ g/ml TTC	50 μ g/ml TTC
100	0	2
500	0	24
1000	0	24
10,000	0	not able to count

Platings were done in duplicate on sorbose minimal agar. Plates were incubated 72 hours at 30 C. Smear plate technique was used on the Emerson a wild type.

there are both nuclear and cytoplasmic determinants for TTC resistance in Neurospora crassa. Because we had already noted a correlation between mating type and TTC resistance or sensitivity, reciprocal crosses of OR-SL A and a strains (Table 5, Crosses V and VI) were done. There was a 1:1 segregation of TTC resistance and TTC sensitivity, indicating a single gene difference. That this gene was closely linked to mating type was evidenced by the fact that all A strains were TTC-resistant and all a strains were TTC-sensitive.

In order to learn how close the TTC locus was to mating type, 206 random ascospores were isolated in addition to the

TABLE 5

SEGREGATION OF RESISTANCE AND SENSITIVITY TO TTC IN
CROSSES OF OAK RIDGE-ST. LAWRENCE STRAINS

Cross No.	<u>Parents</u>		No. of Asci	% Germination	<u>Isolate Response to TTC</u>			
	Protoperi- thecial	Conidial			<u>A</u> <u>TTC^r</u>	<u>A</u> <u>TTC^s</u>	<u>a</u> <u>TTC^r</u>	<u>a</u> <u>TTC^s</u>
V	OR-SL <u>a</u> <u>TTC^s</u>	ST <u>A</u> <u>TTC^r</u>	5	100	20	0	0	20
VI	ST <u>A</u> <u>TTC^r</u>	OR-SL <u>a</u> <u>TTC^s</u>	4	97	15	0	0	15
XXVII	ST <u>A</u> <u>TTC^r</u>	NC-SL <u>a</u> <u>TTC^r</u>	5	100	20	0	20	0
XXVI	NC-SL <u>a</u> <u>TTC^r</u>	ST <u>A</u> <u>TTC^r</u>	4	95	16	0	14	0
XLVII	ST <u>A</u> <u>TTC^r</u>	SL <u>a</u> [<u>mi-1</u>]	3	58	6	0	0	8
XLIX	ST <u>A</u> <u>TTC^r</u>	SL <u>a</u> [<u>mi-1</u>], <u>nic-2</u> (?)	3	83	8	0	0	12

Sensitivity or resistance to TTC determined by spot test, incubated at 30 C and scored at 24-48 hours.

ordered tetrads listed in Cross V. Genetic analyses of the random isolates revealed only 2% recombinants--all of them TTC-resistant a. The other nonparental ditype, TTC-sensitive A, was not found. If we assume that this ditype does exist, but was missed in the relatively small sample, then there would be 4% recombinants, indicating a distance of two map units between mating type and the TTC locus in the OR-SL wild type.

The TTC-resistant a strains could have been the result of either reversion or recombination. At first it was felt that they were most likely the result of a reversion since almost the same per cent of colonies appeared in the sorbose plating of a TTC-sensitive a. However, while observing a conidia on a sorbose-TTC-agar film, we noted that hyphal fragments regenerated while conidia did not germinate under the same conditions. This suggested that, despite filtering through sterile glass wool, some hyphal fragments could have been plated and given rise to colonies. Because colonies are not usually counted under a microscope, the origin of each colony would be difficult to establish. The colonies which did develop in the sorbose plating of a TTC-sensitive a wild type were isolated. After they had conidiated, they were spot tested, and all showed definite zones of inhibition. It would seem then that these occasional colonies are indeed the result of regenerating hyphal fragments. This in turn strengthens the possibility that the nonparental ditypes

found in the OR-SL crosses are recombinants.

When reciprocal crosses of Emerson 5256 A and Emerson a (Emerson a being an isolate from a cross of Emerson 5256 A and 5297 a) were done, the two classes of nonparental ditypes were found. Emerson 5256 A was TTC-resistant and Emerson a was TTC-sensitive, following the pattern of the OR-SL strains. When nine ordered asci from these reciprocal crosses were isolated, eight gave the expected segregation of all a TTC-sensitive and all A TTC-resistant. However one ascus gave us the nonparental ditypes, a TTC-resistant and A TTC-sensitive, as well as the parental ditypes (a tetratype tetrad). The relative ease with which both recombinants were found among the few progeny tested from the Emerson crosses suggests two possibilities. One possibility is that, by chance, the ascus with recombinants in the isolation of Emerson crosses was found while a similar one in the OR-SL crosses was missed in the isolation. This is still possible despite the fact that more isolates from the OR-SL crosses were tested; we may not have done enough.

Another explanation could depend on the location of the gene controlling the resistance of Emerson strains to TTC. There was a 1:1 segregation of TTC-resistance to TTC-sensitivity when Emerson A and a were crossed. In most cases the A isolates were resistant, and the a progeny were sensitive. The one exception was the ascus just described. Even this ascus displayed a 1:1 segregation of TTC-sensitivity to TTC-resistance so the Emerson strains do have a gene controlling

response to TTC. In these strains, however, it could be located farther from the mating type locus than it is in the OR-SL strains. This situation would allow more crossing over resulting in the recovery of the nonparental ditypes.

To test this possibility all ordered asci that had ever been isolated in this laboratory were analyzed, including only those **which** were from intrastrain crosses. Based on the relative numbers of 1st and 2nd division segregations we found that in the RL strains the mating type locus fell about 2.7 map units from the centromere. In the OR-SL the locus was 5.5 map units from the centromere, but in the Emerson strains it was 21 map units from the centromere (Wilson and Wallace, unpublished). So it does indeed appear that in the Emerson strains we are dealing with a mating type locus which is quite some distance from the centromere, and therefore farther from the TTC locus. Although no difficulty was encountered in isolating TTC mating type recombinants from intrastrain crosses of Emerson wild types, interstrain crosses of Emerson and OR-SL strains yielded no recombinants in five ordered asci and 100 random isolates.

When a number of randoms from OR-SL strains were isolated as previously noted, a TTC-resistant strains were recovered but no A sensitive ones. Again a large enough sample may not have been isolated or the A mating type with the allele for TTC sensitivity may be lethal in the OR strains. A low per cent of germination with more A than a ascospores

not appearing would make this explanation seem plausible. However, there was almost 100% germination in the crosses in question and random ascospores were about half A and half a. On occasion an ascus has been found in which all the A isolates did not germinate. But the a's did and were sensitive so the A's should have been resistant.

Evidence has been presented of a single gene determinant for the response of *Neurospora* strains to TTC. Two alleles of this gene have been discussed--a resistant and a sensitive. They are linked to mating type and therefore are located in linkage group I. We proposed that these alleles be designated TTC^r for resistance to TTC and TTC^s for sensitivity to TTC. The guidelines for *Neurospora* nomenclature (Barratt and Perkins, 1965) suggest this system for sensitive and resistant responses since it is not clear which response is that of the wild type and which is the mutant response. Both the OR-SL and Emerson strains show a 1:1 segregation of TTC^r to TTC^s, as already mentioned. The third major *Neurospora* wild type tested for TTC response was Rockefeller-Lindegren, and it presented its own set of problems.

Neither mating type of the Rockefeller-Lindegren strains was inhibited by TTC. There are a number of possible explanations for this behavior:

- 1) the TTC^r allele is present in both mating types.
- 2) more than one gene is involved in this TTC resistance.
- 3) there is cytoplasmic control of resistance.
- 4) some combination of 1, 2, and 3 is responsible.

Two sets of reciprocal crosses between the OR-SL and RL strains were made to test the above hypotheses. Analyses of these crosses (Table 6) eliminate cytoplasmic inheritance as a factor in the resistance of the RL strains and suggest strongly that hypothesis 1 is correct. The cytoplasm is eliminated as a factor in RL resistance to TTC because there was no difference in the pattern of TTC resistance in the progeny from the reciprocal crosses. Previous results of reciprocal crosses of the OR-SL strains showed plainly that in these strains there is no cytoplasmic inheritance of TTC resistance (Table 5). Therefore, in cross XLI (Table 6), $RL^{\circ} \times OR-SL^{\sigma}$, all possibility of cytoplasmic inheritance is eliminated since it was not transmitted by the conidial parent, and there were no cytoplasmic factors in the protoperithecial parent. If the resistance of RL a were due solely to a cytoplasmic factor, there would have been a 1:1 segregation of resistance to sensitivity in the progeny.

Since all progeny of the reciprocal crosses (Table 6) were resistant to TTC and the resistance segregated strictly according to mating type, it appears virtually certain that hypothesis 1 describes the actual situation in the RL strains. RL a possesses the same allele for TTC resistance as RL A, and this allele is probably the same as that of OR-SL A. Any other possibility, e.g. TTC sensitivity being controlled by another gene, would have resulted in segregation of that gene in at least two of the crosses.

The resistance to TTC in the RL a strain used in these

TABLE 6

SEGREGATION OF RESISTANCE AND SENSITIVITY TO TTC IN CROSSES OF
OAK RIDGE-ST. LAWRENCE AND NC ROCKEFELLER-LINDEGREN STRAINS

Cross No.	<u>Parents</u>		No. of Asci	% Germination	<u>Isolate Response to TTC</u>			
	Protoperi- thecial	Conidial			<u>A</u> <u>TTC^r</u>	<u>A</u> <u>TTC^s</u>	<u>a</u> <u>TTC^r</u>	<u>a</u> <u>TTC^s</u>
XL	ST <u>A</u> <u>TTC^r</u>	NC-RL #20 <u>a</u>	5	100	20	0	20	0
XLI	NC-RL #20 <u>a</u>	ST <u>A</u> <u>TTC^r</u>	5	100	20	0	20	0
LXVIII	OR-SL <u>a</u> <u>TTC^s</u>	NC-RL <u>A</u> <u>TTC^r</u>	5	82	18	0	0	16
LXVII	NC-RL <u>A</u> <u>TTC^r</u>	OR-SL <u>a</u> <u>TTC^s</u>	5	88	16	0	0	19

Sensitivity or resistance to TTC determined by spot test, incubated at 30 C and scored at 24-48 hours.

crosses could conceivably have been peculiar to it and not indicative of TTC response of RL strains in general. Accordingly, 17 Rockefeller-Lindegren strains were spot-tested, from the original Lindegren 1A and 25a through 5 generations of progeny. Every strain, regardless of mating type, was TTC^r, proving that our results with the two NC-RL strains crossed were valid.

The [mi-1] strains from OR-SL and RL backgrounds show different tolerance levels with regard to TTC. The OR-SL [mi-1] strains were inhibited at a concentration which did not affect the [mi-1] strains from the RL background. When an OR-SL A was crossed as the protoperithecial parent to an OR-SL [mi-1] a there was a 1:1 segregation of TTC-resistance to TTC-sensitivity. The results are given in Table 5 Cross XXVI. This confirms the presence of the TTC^s allele linked to the a of the OR-SL [mi-1]. Despite this TTC^s the [mi-1] was still resistant to TTC when sorbose plated, although not at as high a concentration as an RL [mi-1] which has the TTC^r allele linked to a. The fact that the OR-SL [mi-1] strain was still resistant lends support to the hypothesis that there is cytoplasmic as well as nuclear resistance to TTC in the [mi-1] strains of Neurospora crassa.

No [mi-1] strains tested were inhibited by the minimum TTC concentrations which prevented the growth of OR-SL a TTC^s conidia. When an [mi-1] was shown to possess the TTC^s allele, it could still tolerate 50 µg/ml TTC. At this concentration the wild type was completely inhibited. If [mi-1]

also had the TTC^R allele, it could withstand more than 100 µg/ml TTC. Whatever causes the [mi-1] strains to be resistant, it is not the same as that responsible for the A/a pattern because the resistance is shown to be additive. Table 7 shows the per cent inhibition of various strains in the presence of a number of TTC concentrations. An OR-SL A TTC^R is listed as a basis for comparison. The a TTC^R is included as well as two [mi-1] strains, one from the OR-SL background and one from the RL background. The A and a wild types with the TTC^R allele exhibited almost the same pattern of inhibition, although the a was still inhibited somewhat at 50 µg/ml. The RL [mi-1] showed even less inhibition than these two because of the added cytoplasmic resistance. The nuclear sensitivity of the OR-SL [mi-1] is very evident in the concentration level of TTC which it can tolerate. It was resistant to 50 µg/ml, but at 75 µg/ml had already succumbed partially, and at 100 µg/ml was completely inhibited.

Two RL strains are included in this table; one is an A and the other is a. The A mating type was not inhibited up to a concentration of 150 µg/ml TTC. At this concentration 7% of the conidia were prevented from germinating. In general, above a concentration of 200 µg/ml, the NC-RL A was inhibited much less than any resistant OR-SL strain. The NC-RL [mi-1], however, showed an even greater decline in inhibition up to a concentration of 600 µg/ml. This strain has the cytoplasmic resistance of [mi-1] in addition to the RL resistance, so we expected it to be capable of germinating

TABLE 7

EFFECT OF TTC CONCENTRATIONS ON TTC-RESISTANT STRAINS

Concentration TTC ($\mu\text{g/ml}$)	% Inhibition					
	NC-RL <u>a</u>	NC-RL <u>A</u>	ST <u>A</u>	NC-SL <u>a</u> <u>TTC^r</u>	NC-RL <u>a</u> [<u>mi-1</u>]	OR-SL <u>a</u> [<u>mi-1</u>]
600	78	76	98	99	93	100
500	73	83	97	96	51	100
400	47	48	96	98	41	100
300	60	34	69	73	0	100
200	22	24	36	60	0	100
150	5	7	14	29	12	100
100	3	0	0	24	0	100
75	5	0	12	3	-	83
50	10	0	2	19	0	0

Inoculated with approximately 100 conidia and incubated at 30 C for 120 hours. Per cent inhibition is based on number of colonies appearing on control plates containing no TTC.

in the presence of higher concentrations of TTC.

The NC-RL a had approximately the same resistance as that exhibited by the NC-RL A. The slight variations could easily have been the result of plating differences. For example, there could have been more aerial hyphae in one or the other. The a from the RL background was more resistant than the strains from the OR-SL background. Neither the OR-SL A nor the OR-SL TTC^r a were as resistant as the RL a. This resistance is illustrated in Figure 1. Three a strains and two A strains are included. Since we have found no TTC^s A strains in the OR-SL background this type could not be tested. The OR-SL TTC^r a is included, and while it was more resistant than the OR-SL a strains, it was not as resistant as the RL a strain or the OR-SL A at lower concentrations.

The inheritance of the response of Neurospora to TTC still poses questions which will have to be answered. As already mentioned, the TTC response of isolates is routinely determined by spot tests. Without this technique we would probably still be sorbose plating isolates from the first crosses. Figure 2 is a photograph of a typical spot test plate. One plate will hold the contents of one ascus plus two control strains, generally the parents or a known A-TTC^r and a-TTC^s. The plate pictured has been incubated at 30 C for 24 hours. The a control and 4 ascospores show clearly the inhibitory effect of TTC, while the A and the 4 remaining ascospores exhibit their resistance.

Figure 1. PATTERNS OF TTC INHIBITION IN WILD-TYPE STRAINS OF NEUROSPORA

Inoculated with approximately 100 conidia and incubated at 30 C for 120 hours. Per cent inhibition is based on number of colonies appearing on control plates containing no TTC.

STRAIN

ug/m

RL

OR-SL

TTC^r

OR-SL

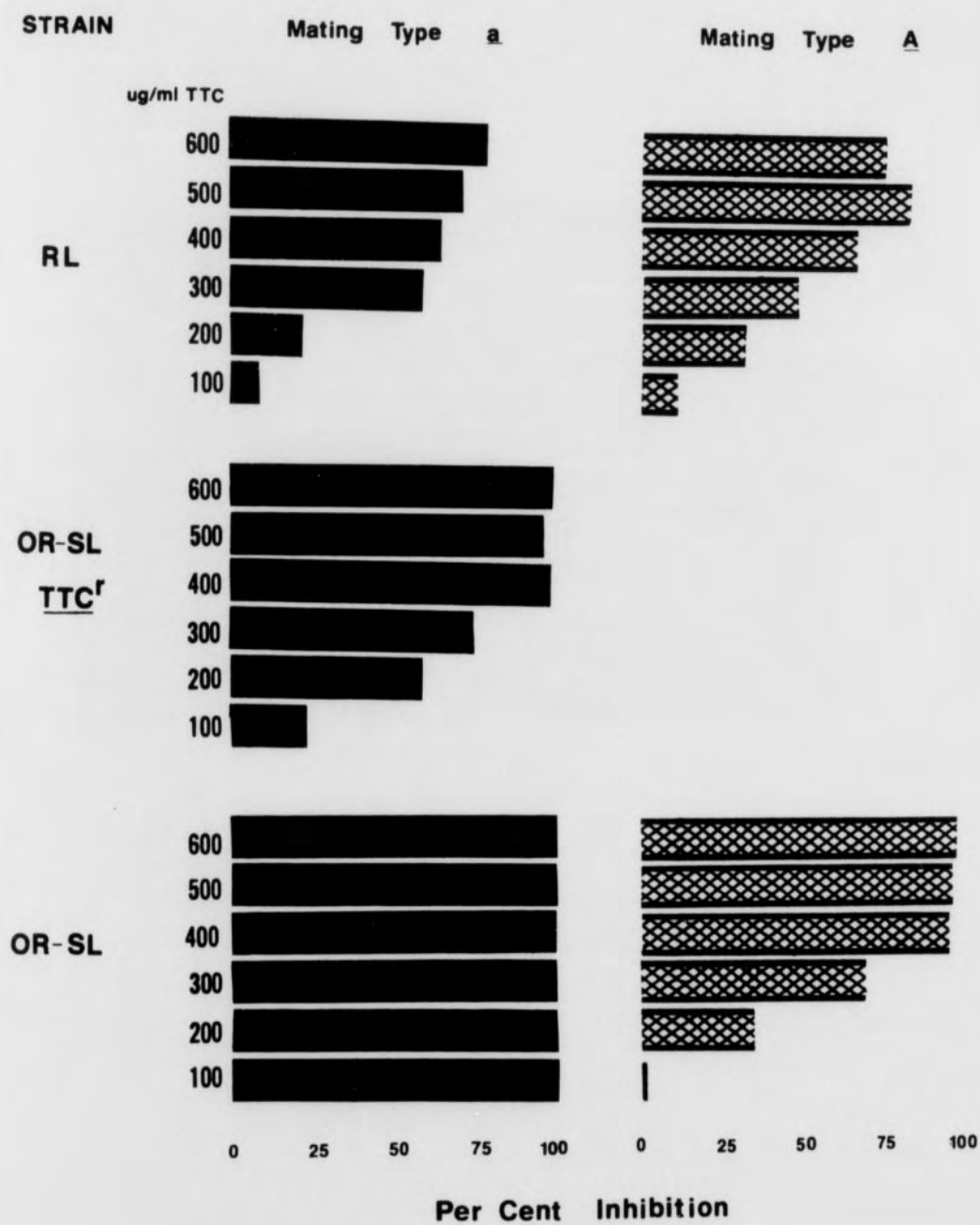
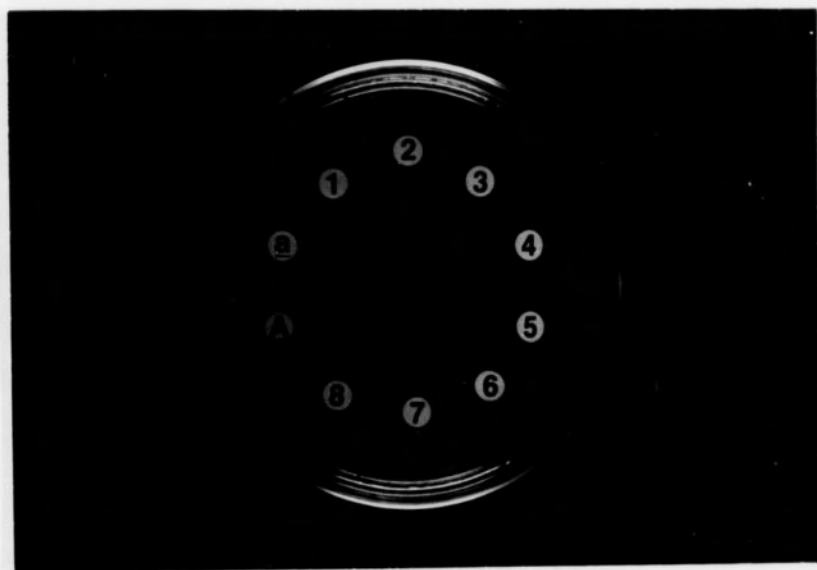


Figure 2. TTC SPOT TEST PLATE

Segments 1-8 are the 8 ascospores of one complete ascus. Segment a is the a parent, A is the A parent. Isolates have been previously sex-tested. White dots are the discs impregnated with 10 mg/ml TTC. This plate has been incubated at 30 C for 24 hours. All numbers and designations have been superimposed on the discs on the print to facilitate identification. In actual tests the number is placed randomly on the segment with a wax pencil.



Experiments on the Mechanism of TTC Resistance

At this point it was known that a single gene controlled TTC response in most wild type strains, and this was combined with a cytoplasmic resistance in [mi-1]. In all of the experiments conidia were used as the inoculum, so a series of three experiments was devised to determine how the TTC was affecting these conidia. Since only the a mating type was inhibited by TTC, the OR-SL a wild type and the Emerson a wild type were used.

We set out to find: 1) if the physiological age of conidia were a factor in reaction to TTC, 2) if the conidia were killed or inhibited by TTC, and 3) if the presence of sorbose had any effect on the survival rate of susceptible conidia. Basically an agitated conidial suspension in a nutrient medium was incubated at 25 C. The incubation flasks were 500 ml. Erlenmeyer flasks containing 150 ml of the appropriate liquid medium. The flasks were inoculated with approximately 75,000 conidia, and either 0.1 or 0.2 ml portions were removed and sorbose-plated at timed intervals. For 50 conidia per plate 0.1 ml was used and for 100 conidia a 0.2 ml fraction was plated.

In order to determine the stage of germination at which TTC was effective, samples were taken at 0, 2, 4, 6, and 10 hours from a flask of Vogel's minimal medium inoculated with conidia and incubated at 25 C. The samples of conidia were streaked over the surface of the plates containing sorbose

and TTC. However, in later work the conidia were added to the medium while it was held at 47 C in a water bath. The screw-capped tubes were inverted once, then poured immediately. The petri dishes were then swirled to give a very satisfactory distribution of conidia. The previously used smear-plate technique promoted the clumping of conidia and also presented more opportunity for contamination, therefore, subsequent experiments were done using this pour-plate technique. The results of this experiment are given in Table 8. The TTC successfully inhibited the conidia until they had been incubated for six hours. As the conidia germinated, the per cent inhibition decreased, indicating that the conidia themselves were affected and not the hyphae of the microcolony. This explanation is supported by the information already presented that hyphal fragments could regenerate while conidia under the same conditions failed to germinate.

The second experiment was to determine what effect incubation with TTC would have on the per cent survival of a TTC-sensitive a wild type and to find out whether TTC merely inhibited or actually killed the conidia. Two flasks of Vogel's minimal medium were inoculated and incubated at 25 C on magnetic stirrers. One flask contained 100 µg/ml TTC; the other did not and thus served as a control. Samples from each were plated at one-hour intervals for 4 hours. Table 9 gives the results of this experiment. From the low per cent of ungerminated conidia (% inhibition), it is evi-

TABLE 8

EFFECT OF PHYSIOLOGICAL AGE OF GERMINATING CONIDIA
ON TTC SENSITIVITY

Hours Incubated	% Inhibition
0	97
2	97
4	100
6	77
10	23

Conidia of Emerson a incubated at 25 C in Vogel's minimal medium with continuous stirring. Samples containing approximately 50 conidia sorbose plated at indicated intervals; TTC concentration--100 µg/ml. Per cent inhibition based on number of colonies appearing on control sorbose plates with no TTC.

TABLE 9

EFFECT OF INCUBATION WITH TTC ON VIABILITY OF CONIDIA

Hours Incubated	% Inhibition
0	3
1	2
2	0
3	6
4	0

Conidia of OR-SL a incubated at 25 C in two flasks of Vogel's minimal medium with continuous stirring. Concentration of TTC in experimental flask--100 µg/ml. Samples containing approximately 100 conidia from each flask plated at indicated intervals on sorbose minimal medium. Per cent inhibition based on number of colonies appearing on plates inoculated with sample from control flask containing no TTC.

dent that the conidia were merely inhibited and not killed. If they had been killed none would have germinated when plated on sorbose. Once away from the TTC, the conidia apparently continued their arrested development, indicating the TTC inhibition is reversible.

There was still the possibility that the TTC produced its effect only when it was associated with sorbose. The third experiment consisted of two flasks: one of Vogel's minimal medium and one of sorbose minimal. After inoculation and before adding TTC, samples were plated from each flask to serve as controls. The TTC (100 $\mu\text{g}/\text{ml}$) was added to each flask, and the flasks were incubated with stirring for 72 hours. Samples were plated on sorbose with no TTC at 24-hour intervals. The data from this experiment are presented in Table 10. There was some increase in inhibition when conidia were incubated with TTC in the presence of sorbose.

To directly observe the conidia germinating on sorbose with and without TTC, microcultures were prepared using standard sorbose minimal medium to coat the cover slips. The required concentrations of TTC were added to the media before the cover slips were coated. The concentrations used were 50, 100, and 1200 $\mu\text{g}/\text{ml}$. A loop of conidial suspension was touched to the agar-coated surface which was then blotted with filter paper to anchor conidia and remove excess water. The preparations were incubated at 30 C and observed at various intervals. To observe the germinating conidia the microcultures were placed face down on a deFonbrune oil chamber

TABLE 10
EFFECT OF SORBOSE ON CONIDIA INCUBATED WITH TTC

Hours Incubated	% Inhibition	
	Sorbose + TTC	Vogel's minimal + TTC
0	0	0
24	16	0
48	86	56
72	97	80

Conidia of OR-SL a incubated at 25 C in media with continuous stirring. Concentration of TTC--100 µg/ml. Samples containing approximately 100 conidia plated at indicated intervals on sorbose minimal medium. Per cent inhibition based on number of colonies appearing on plates inoculated with a sample from each flask before TTC was added.

which was then filled with sterile silicone oil, 50 centistokes viscosity (Dow Corning Co.). Photographs were taken of several preparations.

Wild type A and a and an [ml-1] a, all from the SL background were observed. Although preliminary observations were done with silicone oil in the chamber, 15% sucrose-Vogel's minimal medium was used for later work. As previously noted, this medium is necessary for continued development of the conidia, and they should be in a favorable environment. While the silicone oil does not harm the organism, it does not encourage growth.

In general, it was found that in a wild type, conidia failed to germinate and developed large abnormal vacuoles in the presence of 100 µg/ml TTC. When A wild type was observed

on 1200 $\mu\text{g/ml}$ TTC, the ungerminated conidia again contained large vacuoles. This concentration of TTC is more than double the amount that an A TTC^r can tolerate. When the concentration was dropped to 120 $\mu\text{g/ml}$ the A did grow which is what it should have done. In control microcultures of sorbose without TTC, this vacuolation was not noted, and conidia germinated. The [mi-1] did not germinate in the presence of 100 $\mu\text{g/ml}$ TTC and were vacuolated. Again conidia on control microcultures germinated. When the [mi-1] conidia were put on 50 $\mu\text{g/ml}$ TTC they did germinate. This behavior was expected since the SL [mi-1] strains can only tolerate this concentration on a sorbose plating. A few ungerminated conidia were found and these had multiple small vacuoles. The [mi-1] conidia tended to form multiple germ tubes while the wild type strains did not.

The five photographs in Figure 3 are the OR-SL A and a strains on microcultures of sorbose and TTC. In picture (a) the a conidia have only been exposed to TTC for 5 hours but already small vacuoles are visible. After they had been in the presence of TTC for 24 hours, the small vacuoles had coalesced to form one large outstanding vacuole as shown in picture (b). These inclusions were not nuclei; no nucleoli were present. Unfortunately, after 24 hours at 30 C, a conidia will have germinated and literally overrun the cover slip so no control photograph of a at 24 hours is included. However, picture (c) can serve as a control for the normal



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Figure 3. OR-SL WILD TYPE A AND a MICROCULTURES GROWN ON
SORBOSE MINIMAL MEDIUM WITH AND WITHOUT TTC

Incubated at 30 C.--(a)OR-SL a 100 μ g/ml TTC 5 hours.--
(b)OR-SL a 100 μ g/ml TTC 24 hours.--(c)ST A no TTC 6.5 hours.--
(d)ST A 120 μ g/ml TTC 5 hours.--(e)ST A 1200 μ g/ml TTC 20
hours.

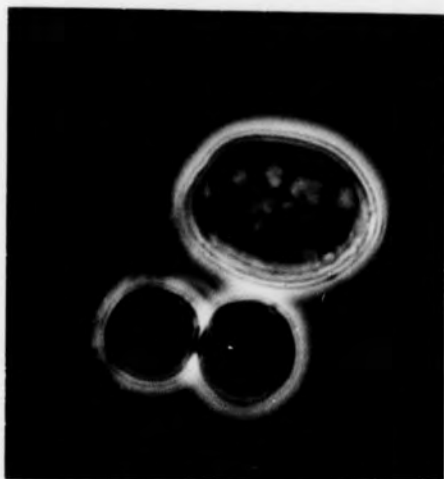
Magnification - (a)-(d) - 2000 X
(e) - 1600 X



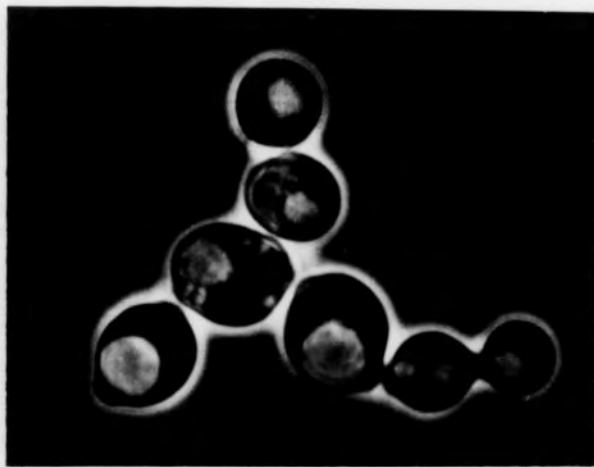
(a)



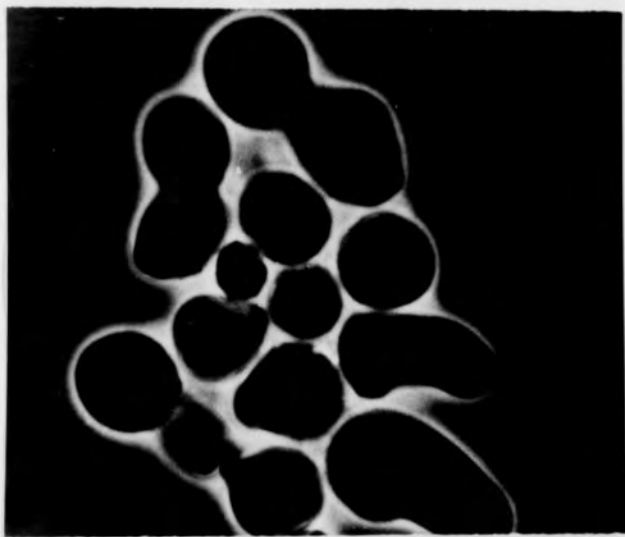
(d)



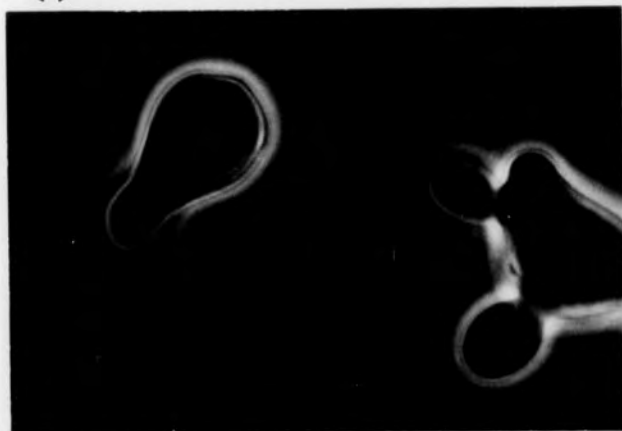
(a)



(b)



(c)



(d)



(e)

appearance of conidia germinating on sorbose after incubation at 30 C for 6.5 hours. The strain is A, and there is no TTC in the sorbose agar film. The nucleoli are discernible within the nuclei. When A conidia were incubated on sorbose agar containing 120 $\mu\text{g/ml}$ TTC, they still germinated, as expected from the results of sorbose platings. None of the typical but abnormal vacuoles are in evidence in the germinating conidia in picture (d). However, when the TTC concentration was increased to 1200 $\mu\text{g/ml}$ as in picture (e), the large abnormal vacuoles again appear. The conidia which show this vacuolation do not germinate. An interesting observation about this microculture concerns the time. Picture (e) was taken after the conidia had been incubated for 20 hours. Despite the length of the incubation period and the high concentration of TTC, the A conidia do not have the one large vacuole seen generally in a after about the same incubation period. The A was treated with double the concentration which inhibits it in a sorbose plating and should logically have the dramatically large vacuoles as seen in a treated with inhibiting concentrations of TTC. If these vacuoles were the result of the TTC which has been absorbed by the conidia and if the size can be related to the amount of time the TTC is inside, this experiment could indicate a differential permeability to TTC in mating types. The a in contact with TTC for only 5 hours already has numerous small vacuoles while the A incubated with a TTC concentration high

enough to have the same effect did not. The TTC could take so much longer to penetrate the wall of A that the conidia are in a more favorable survival state by the time the TTC can exert its effect. The flask experiment had previously shown that once conidia reach a certain germination stage they become less susceptible to TTC. The 6th hour plating of incubated conidia showed a decrease in sensitivity to TTC (Table 8). Perhaps in a the TTC gets in before the six-hour stage while in A it takes longer and consequently requires a much higher concentration to be inhibited. This has already been found to be true.

Figure 4 includes three photographs taken of an OR-SL a [mi-1] on sorbose medium-coated cover slips. All were taken after 20 hours incubation at 30 C. Because [mi-1] grows at a slower rate than other strains, the 20 hours here is not equivalent to the same amount of time shown in Figure 3 which is of wild type strains. The conidia in pictures (a) and (b) are germinating. The sorbose agar medium in (a) contained no TTC and is the control microculture for this series. We could not be sure how the [mi-1] a would look when its conidia germinated in the presence of TTC in spite of the observations already made on wild type A and a strains. The [mi-1] carried the TTC^S allele common to a, but it was still resistant to a concentration of 50 µg/ml TTC. When the conidia on 50 µg/ml TTC were observed after 20 hours incubation at 30 C, they had germinated. However, when the conidia were treated

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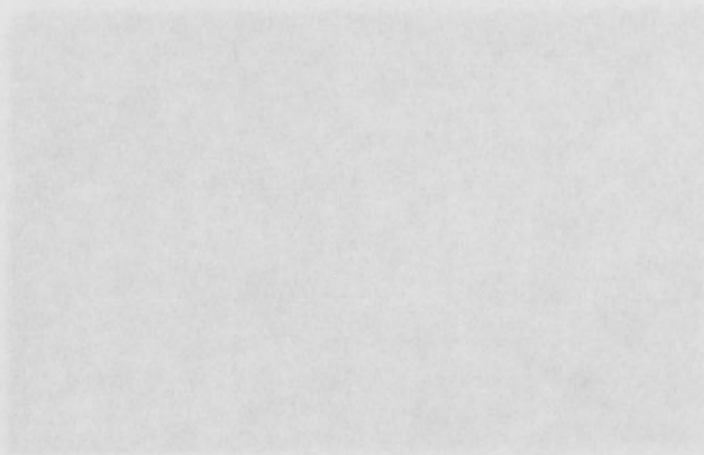
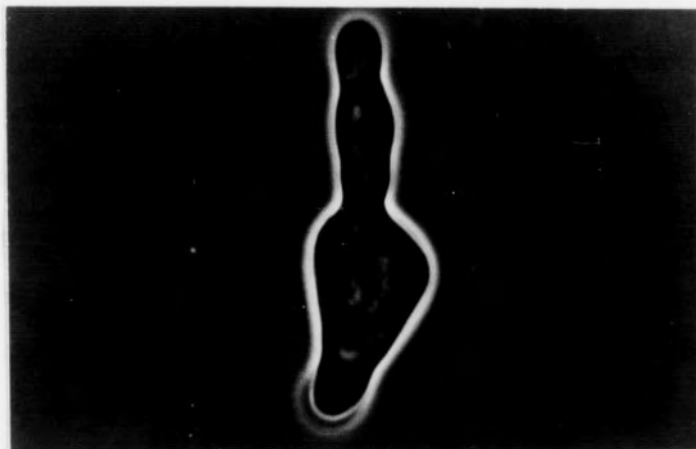


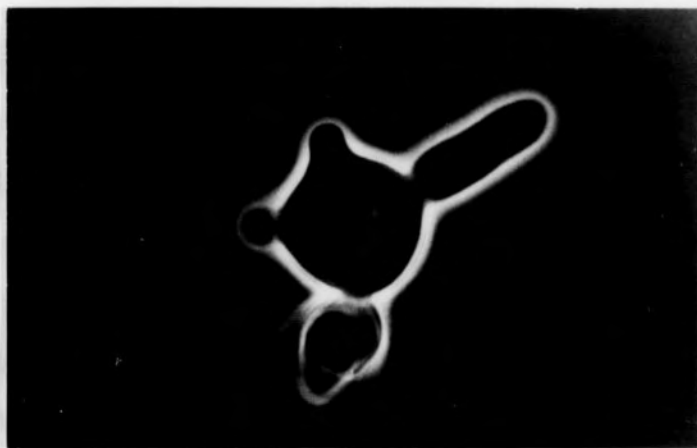
Figure 4. OR-SL [m1-1] a MICROCULTURES GROWN ON SORBOSE
MINIMAL MEDIUM WITH AND WITHOUT TTC

Incubated at 30 C.--(a)OR-SL [m1-1] a no TTC 20 hours.--
(b)OR-SL [m1-1] a 50 μ g/ml TTC 20 hours.--(c)OR-SL [m1-1] a
100 μ g/ml TTC 20 hours.

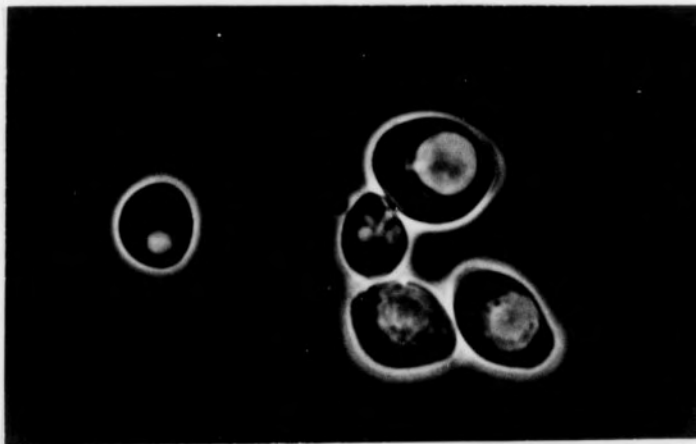
Magnification - 1600 X



(a)



(b)



(c)

the same way in the presence of twice the concentration of TTC, they failed to germinate. As picture (c) shows they were not only inhibited, but the typical vacuolation found in a TTC^S a wild type was in evidence. It almost seems as if the higher concentration of TTC somehow overwhelmed the cytoplasmic resistance of the [mi-1] strain and in doing so allowed the TTC^S allele of a to show its presence.

From even these few observations we can say that a concentration of TTC which will completely inhibit a strain in a sorbose plating will produce the abnormal vacuolation in the ungerminated conidia. When the TTC concentration present is one which will not inhibit the strain in a sorbose plating, the conidia will germinate and do not show any abnormal vacuolation. The vacuolation first appears as many small vacuoles eventually coalescing into a very large vacuole. No crystals of formazan were seen in any of the strains tested. TTC could not be injected into conidia to prove that response to TTC is a permeability phenomenon, but a similar approach using microcultures consisting of hyphae as are used for microinjection could be employed. TTC could be put in the medium used to fill the chamber, and it could be injected as well.

Brock (1958) found that TTC was reduced by yeast strains which were inhibited by it as well as those which were not. In addition, he found formazan present in both uninhibited and inhibited strains. As a result of these observations,

he theorized that permeability was not a factor in the inhibition effect of TTC. Nevertheless, we felt that the mating type difference in response to TTC could be more plausibly explained by a difference in membrane permeability than by a basic difference in metabolism of the A and a strains. The permeability difference hypothesis was favored because it fitted well with the evidence of a single gene for TTC response. The TTC gene could determine membrane permeability. It did not seem likely that the two mating types would have different metabolisms.

The following experiments were carried out in an attempt to elucidate the mechanism of response to TTC in *Neurospora*.

Using microcultures, (Wilson, 1961) the hyphae of the two mating types were observed in deFonbrune oil chambers (W. H. Curtin and Co.). In order to by-pass the possibility of variation in membrane permeability, TTC was injected into the hyphae of wild type A and a strains and [mi-1]. The TTC concentration was 10 mg/ml in 0.1M phosphate buffer at pH 6.8. The buffer was used for injections since it does not disturb the cell. The experiment placing TTC outside the hyphae was done with a 15% sucrose solution, which is slightly hypertonic, to prevent bursting of hyphal tips; the Vogel's minimal was added to promote growth and regeneration if it was possible.

The following relationship has been determined between the concentration of a substance (in this case, TTC) which

must be injected into a cell and the concentration which would be the equivalent in the microchamber. Assuming uptake at 100%, each cell in the microculture would then contain a concentration of TTC equal to the amount injected by displacing 20% of the cell with a 10,000X concentration of TTC (Bates and Wilson, unpublished).

When the 10 mg/ml TTC solution was injected into A and a wild type, there was no difference in response. In both there was septal formation, and regeneration or flow through the cell indicating that the septal pores were open. When a cell has been injured chemically or physically, one of the indications of impending death is the formation of a plug at the septal pore distal to the injection site. The facts that no plugs formed, septa were laid down and regeneration occurred are all evidence that the injection of even that high a concentration had no detrimental effect on either mating type. This lack of difference in TTC injection response strongly suggests that the dramatic variation in germination of the A and a in the presence of this tetrazolium salt may be the result of a mating type difference in membrane permeability.

When an OR-SL [mi-1] strain was injected with 10 mg/ml TTC, its response was the same as that of wild type. Since the [mi-1] strains are respiratory-deficient and have an altered cytochrome complement, some variation was expected in the effect of TTC on the organism or possibly a difference in the fate of the TTC injected. The [mi-1] strains have an excess of cytochrome c which is the reduction site for TTC. It

would seem plausible to expect a higher rate of formazan production, but there was no dramatic increase in formazan accumulation. This raises an interesting question about the [mi-1] strains. Can they be transporting electrons via another system (and using only a fraction of the cytochrome c), thereby not providing any more reduction potential than a wild type?

Assuming uptake of 100% and using the relationship already noted, a concentration of 1 µg/ml in the chamber should be the equivalent of these 10 mg/ml injections. However, we knew this concentration would probably not be high enough, and when a concentration of 50 µg/ml was used in the chambers of an A, a and [mi-1] (all OR-SL background), there was no sign of any response. If there were a difference in the permeability of the membranes of these three types, some evidence of it should have been noted in these "chamber" experiments. Concentrations of 2.0 mg, 1.5 mg, and 1.0 mg/ml TTC in 15% sucrose--Vogel's minimal medium were tried in the microchambers. The microcultures were incubated at 30 C and observed at various intervals.

The lowest concentration (1.0 mg/ml) of TTC used in the chamber produced no startling or dramatic effect in either mating type after incubation for 60 minutes. There were no crystals; the only difference was a greater incidence of hyphal tip death in the a. This unfortunately can very easily be some problem in the strain itself.

When the TTC concentration was increased to 1.5 mg/ml, we noted several phenomena. There were crystals present in dead cells of A and a, but a also had crystals in the adjacent live cells. In addition blue spheres were observed outside of live and dead hyphae in a; the A also had these spheres, but they were more sparse. Even these rather limited responses required 4 hours incubation.

The highest concentration to be tested so far is 20 mg/ml. After 30 minutes, no crystals were found; after 60 minutes, crystals were abundant in several cells of a but not in A. When these were observed at about 2 and 4 hours, the A generally had fewer crystals than a, and both had the blue spheres outside of the hyphae. The [mi-1] in the presence of this concentration after 2 hours incubation resembled A in that it had no crystals in live cells adjacent to dead cells with crystals in them. They also had no blue spheres outside of hyphae, which distinguishes them from the two wild types.

Figure 5 is a photograph of a crystal that has grown in a live cell of the OR-SL a TTC^S strain. The crystals are red; the reduced form of TTC, the triphenyl formazan, is also red. The crystal in this hypha has grown through the septal pore of the cell. The cell has laid down septa and has regenerated into the adjacent dead cell indicating that the presence of the crystal has not caused any abnormal reaction in that cell. If the outer wall of the hypha is examined, the spheres mentioned previously can be seen.

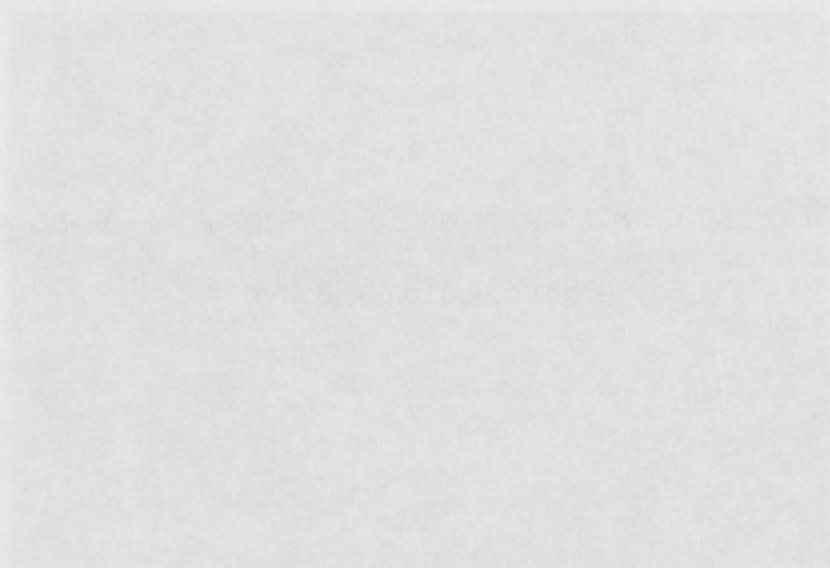
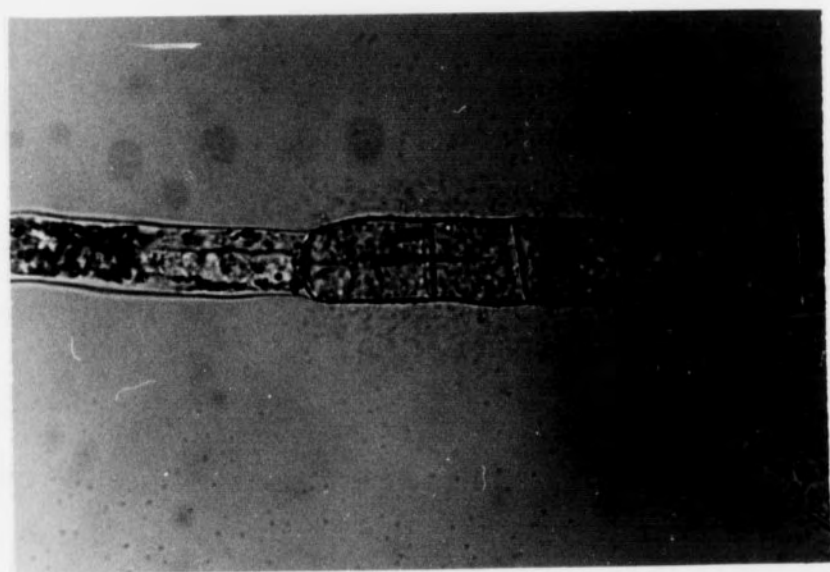


Figure 5. RED CRYSTAL FORMING IN LIVE CELL OF OR-SL a TTC^S

Magnification - 800 X



The possibility that the mating type difference in response to TTC is a membrane permeability phenomenon has been explored. The single gene that is responsible for the strain's reaction could determine some cell membrane difference which would render it either more or less permeable to TTC. On the basis of this information, an A strain which was also TTC^r would have a different cell membrane when compared to a TTC^s. A recombinant should then have an altered cell membrane; an a which was TTC^r should have a cell membrane like that of A TTC^r. In *Neurospora* the cytoplasmic incompatibility reaction is thought to involve the cell membrane (Williams and Wilson, 1966). The cell death associated with this incompatibility closely resembles the mating type incompatibility reaction (Garnjobst and Wilson, 1956). Therefore, the mating type incompatibility could also involve the cell membrane. If this is so, the cell membrane of a TTC^r a should be like that of a TTC^r A, and there should not be an incompatibility reaction when the two fuse.

A TTC^r a was observed microscopically, fusing with a TTC^r A and a TTC^s a. When the TTC^r a and the TTC^r A fused, there was the normal mating type incompatibility reaction with typical vacuolation and dead cells. There was no evidence of the "avoidance reaction" which is a phenomenon whereby hyphae of two strains growing toward each other will start curling away from each other before they are close enough to fuse. There was no lack of fusion either. None of these reactions were observed when the TTC^r a fused with the TTC^s a. Any of

these reactions would have strengthened the possibility of an altered cell membrane in TTC^r a. Not only did they fuse with no problem but there was flow through the interstrain fusions. Apparently, if there were some alteration of the cell membrane of a recombinant, it cannot be detected by compatibility tests. The TTC^r a behaved normally when fusing with other strains. Table 11 summarizes this experiment and its result. The TTC compatibility is an artificial class, there is no evidence that there is any compatibility response connected with the TTC alleles. For simplicity, "+" indicates the same allele; "-" indicates that the different alleles are present in the pair.

Because TTC^r a was a rather rare strain it might have been abnormal when it was crossed. It was crossed to a TTC^r A strain both as the protoperithecial and conidial parent. There were no abnormalities of any sort in the crosses, their isolation, or the isolates themselves. As expected when these isolates were spot tested, all were resistant (+).

Analysis of Heterochondrions with TTC

The TTC resistance of the [mi-1] strains and the complete inhibition of a conidia in the presence of 50 µg/ml TTC have provided one more assay method for the fate of mitochondria isolated from [mi-1] and injected into wild type strains. By sorbose plating this wild type strain which has been injected with [mi-1] mitochondria it is possible to

TABLE 11
MICROSCOPIC EXAMINATION OF FUSION BETWEEN MATING TYPES

Cultures	Compatibility		Observed Results
	Mating Type	"TTC"	
$\text{TTC}^r \underline{a} + \text{TTC}^s \underline{a}$	+	-	+
$\text{TTC}^s \underline{a} + \text{TTC}^r \underline{A}$	-	-	-
$\text{TTC}^r \underline{a} + \text{TTC}^r \underline{A}$	-	+	-

+ = compatibility
- = incompatibility

watch it begin to alter its response to TTC from sensitive as a wild type to resistant as an [mi-1]. From previous experiments it was known that injected [mi-1] mitochondria will displace the normal mitochondria of the recipient strain, resulting in an [mi-1] phenotype. The culture, after a number of serial transfers, will begin to exhibit an [mi-1] growth pattern and rate. The cytochrome spectrum of the recipient assumes the components of an [mi-1] strain and the recipient becomes resistant to TTC (Wilson, unpublished).

Figure 6 is the culmination of all of the experiments described in this paper. By using TTC the takeover by the injected [mi-1] mitochondria can now be traced. This figure consists of two subdivisions, one a control sorbose plating, the other a plating on sorbose with 50 $\mu\text{g/ml}$ TTC. Three different strains were plated under both conditions: the wild type recipient which was serially transferred as a control,

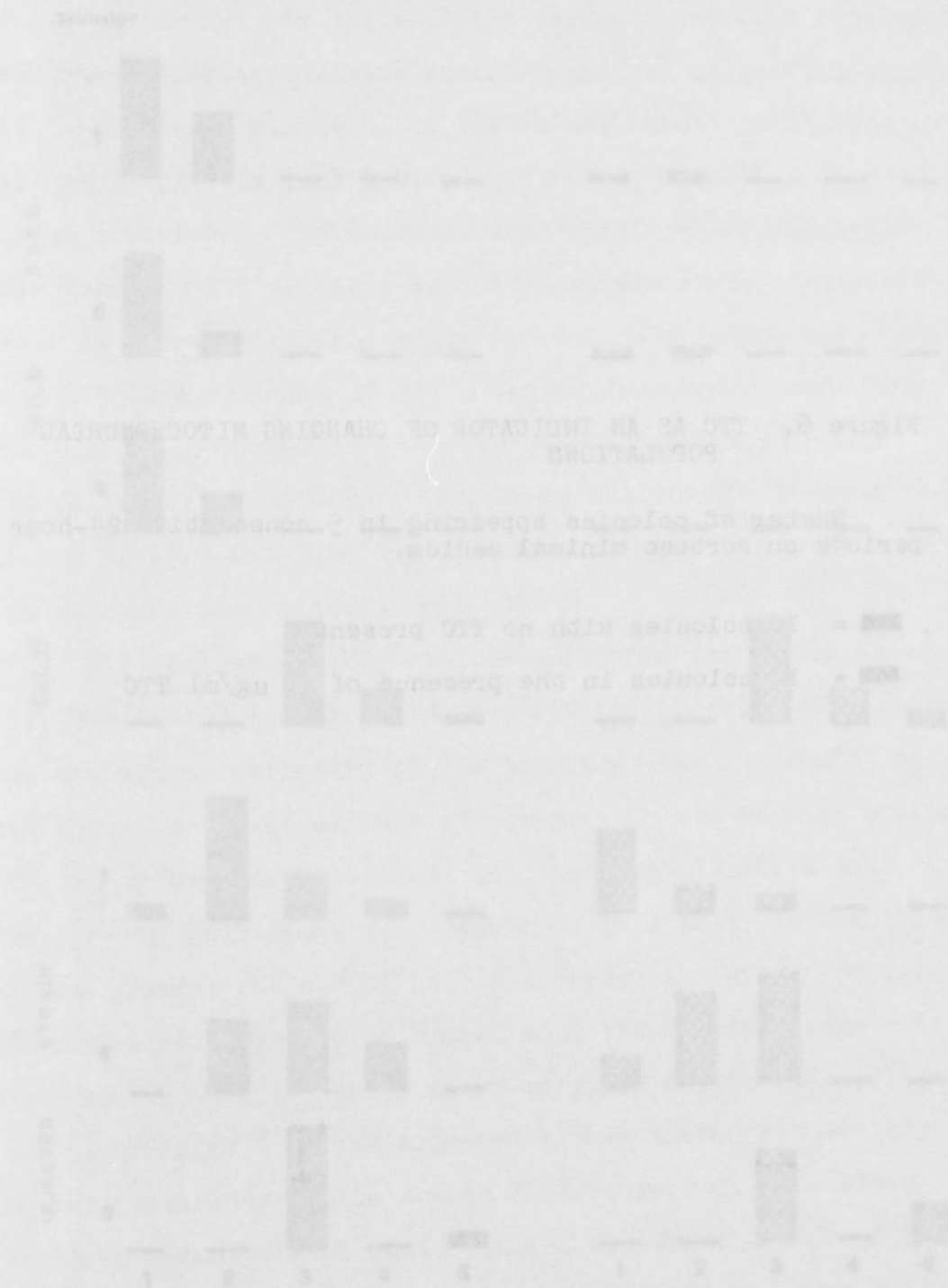


Figure 6. TTC AS AN INDICATOR OF CHANGING MITOCHONDRIAL POPULATIONS

Number of colonies appearing in 5 consecutive 24-hour periods on sorbose minimal medium.

■ = 10 colonies with no TTC present

▣ = 10 colonies in the presence of 50 $\mu\text{g/ml}$ TTC

Transfer

TYPE

WILD

[mi-1]

STRAIN

INJECTED

1

6

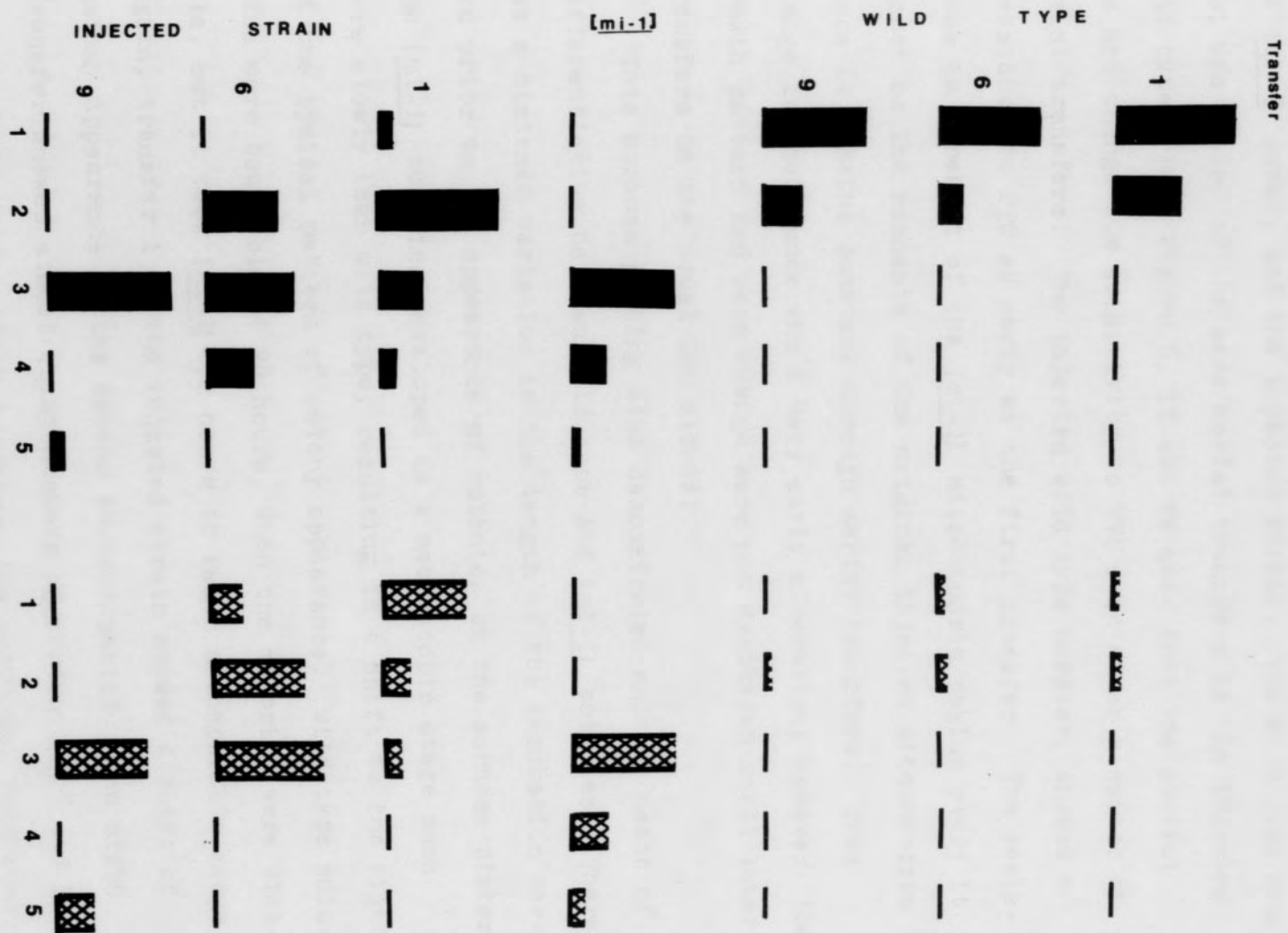
9

1

6

9

1



the [mi-1] donor, and the injected strain. The wild type control was plated at the same serial transfers as the injected wild type. From Figure 6, it can be seen that the control did not change its sensitivity to TTC even after a number of serial transfers. The injected wild type however, showed a resistance to TTC as early as the first transfer. The resistance is a result of the [mi-1] mitochondria taking over; it cannot be the remnants of the original injected mitochondria since it remains constant through serial transfers. This change in resistance was a very early alteration; however, the growth pattern and rate change were not exhibited until later transfers on the usual GSC slants.

This sorbose plating also demonstrated another means of differentiating between wild type and [mi-1] colonies. There was a distinct variation in the length of the incubation period prior to the appearance of colonies on the sorbose plates. The [mi-1] colonies developed to a macroscopic stage much more slowly than wild type, resulting in a shift to the right of the typical pattern of colony appearance. Wild-type colonies were countable at 24 hours, when the majority were visible, but it took [mi-1] 72 hours to reach a comparable stage. Again, transfer 1 of the injected strain showed a shift of colony appearance to the second 24-hour period. The sixth transfer showed almost equal numbers appearing in 48 and 72 hours, while at the ninth transfer, the shift was completely to appearance in 72 hours. The TTC plating illustrates the

same gradual shift to the right, although transfer 1 still had the majority of the colonies appearing in 24 hours. This transfer did show some of the resistance to TTC which is characteristic of [mi-1], but it required a few more transfers before achieving full resistance or a resistance comparable to that of the mitochondrial donor.

Experiments with Other Tetrazolium Salts

As previously mentioned, other tetrazolium salts have been synthesized; among these are nitro blue tetrazolium, neotetrazolium, iodonitrotetrazolium, and thiazolyl blue. Figure 7 shows the reduction sites of the various tetrazolium salts along the electron transport chain in the mitochondria of Jerusalem artichoke tubers (Adapted from Kalina and Palmer, 1968). TTC is reduced at cytochrome c, which is the cytochrome that the [mi-1] strains have in excess. The coincidence was too enticing to disregard, and subsequently [mi-1] was sorbose plated in the presence of several of the tetrazolium salts. A concentration of 50 µg/ml was used for all salts since this concentration had worked for TTC, and according to Nachlas, et al. (1960) by weight their electron-acceptance potentials are about equal. In addition to [mi-1], [mi-2] and [mi-4], which differ from [mi-1] in cytochrome spectrum (Table 12), were tested. To serve as controls a and A wild types were included. All of the strains are from OR-SL background. The [mi-1?] is an injected strain: NC-RL

TABLE 1. REDUCTION STATES OF VARIOUS TETRACARBON ETERS IN THE REDUCTION TRANSPORT SYSTEM

(Adapted from: Kojima and Palmer, 1968. "Reduction of
Tetracarbon Ethers by Zinc and Nickel." *Journal of
Organic Chemistry* 33(1): 1-10)

10	-	Fluorocarbon
20	-	Carbon dioxide
30	-	Carbon monoxide
40	-	Molecular oxygen
50	-	Carbon disulfide
60	-	Carbon disulfide
70	-	Carbon disulfide
80	-	Carbon disulfide
90	-	Carbon disulfide
100	-	Carbon disulfide
110	-	Carbon disulfide
120	-	Carbon disulfide
130	-	Carbon disulfide
140	-	Carbon disulfide
150	-	Carbon disulfide
160	-	Carbon disulfide
170	-	Carbon disulfide
180	-	Carbon disulfide
190	-	Carbon disulfide
200	-	Carbon disulfide

Figure 7. REDUCTION SITES OF VARIOUS TETRAZOLIUM SALTS IN
THE ELECTRON TRANSPORT SYSTEM

(Adapted from: Kalina and Palmer, 1968. "Reduction of
tetrazolium salts by plant mitochondria." Histochemie 14(4):
336)

- Fp - flavoprotein
- CoQ - coenzyme Q
- cyt. - cytochrome
- O₂ - molecular oxygen
- NBT - nitro blue tetrazolium chloride
- TNBT - trinitro blue tetrazolium chloride
- MTT - thiazolyl blue
- INT - idonitro tetrazolium violet
- NT - neotetrazolium chloride
- BT - tetrazolium blue
- TTC - triphenyltetrazolium chloride

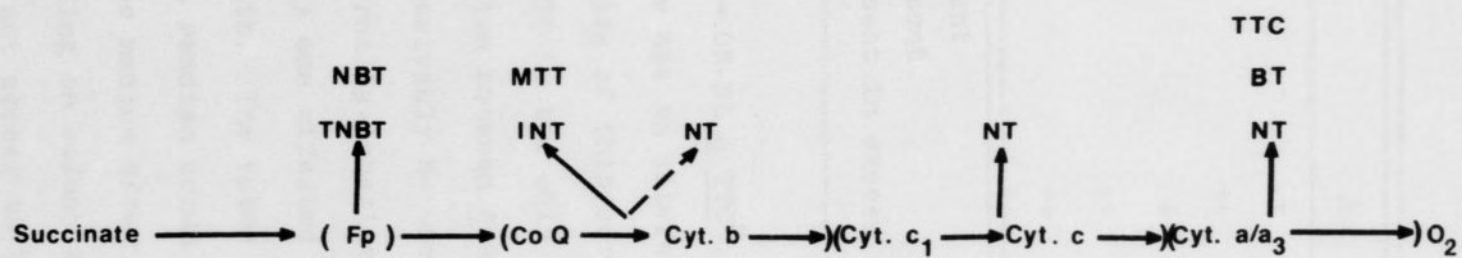


TABLE 12
CYTOCHROME COMPONENTS OF VARIOUS NEUROSPORA STRAINS

Strain	<u>b</u>	<u>c</u>	<u>a/a</u> ₃
Wild type	+	+	+
[<u>mi-1</u>]	-	++	-
[<u>mi-1?</u>]	-	+	-
[<u>mi-2</u>]	+	++	-
[<u>mi-4</u>]	+	++	-

- = cytochrome absent
+ = cytochrome present
++ = cytochrome present in excess of normal amount

[mi-1?] a mitochondria → OR-SL a TTC^S, so it is also an OR-SL strain, but which now has an altered cytochrome complement. Figure 8 shows the results of this sorbose plating. At 50 µg/ml it appears that TTC is the only tetrazolium salt which will allow differentiation between A and a and the [mi-1] strains. INT could conceivably be used to distinguish wild type from the four maternally-inherited mutants. This tetrazolium salt was the only one affected by the sorbose medium held in a 47 C water bath. The tubes of media turned from a caramel brown to a deep reddish brown color. As colonies broke the surface of the medium after the plating, they became red or pink depending on colony age. The other tetrazolium salts tested do not appear to be satisfactory, at

Figure 8. RESPONSE OF OR-SL STRAINS TO VARIOUS TETRAZOLIUM SALTS

All strains are SL background. Wild types incubated at 30 C for 96 hours, [mi-] strains for 120 hours. Inoculum--100 conidia per plate. Plated in triplicate. All tetrazolium salts were 50 µg/ml concentration. Plating medium was sorbose minimal agar. Per cent inhibition based on the number of colonies that appeared on control plates containing no TTC.

■ = 20% inhibition

Strain

NC-OR TTC

NC-OR TTC

























SL [mi-1] T

SL-RL [mi-1]

SL [mi-2] T

SL [mi-4] T

Level of this concentration, for the 5/10/10/10 difference
 size of variation

Strain	% Inhibition			
	TTC	INT	NBT	NT
NC-OR <u>TTC^s</u> <u>a</u>				
NC-OR <u>TTC^r</u> <u>A</u>				
SL (<u>mi-1</u>) <u>TTC^s</u> <u>a</u>				
SL-RL (<u>mi-1?</u>) <u>TTC^s</u> <u>a</u>				
SL (<u>mi-2</u>) <u>TTC^s</u> <u>a</u>				
SL (<u>mi-4</u>) <u>TTC^s</u> <u>a</u>				

least at this concentration, for any systematic differentiation of strains.

DISCUSSION

This work is the first intensive study of the effect of TTC on a filamentous fungus. Weinberg (1953) did include molds in his brief study of selective inhibition caused by TTC, although there are no references to any specific fungi. Brock (1958) interpreted these molds to be filamentous fungi, and although Weinberg merely states that they were not inhibited at the relatively high concentration of 0.25% and little reduced TTC was found in colonies, Brock interpreted this to say that the filamentous fungi are not inhibited by TTC and do not reduce TTC. However, we have found that Neurospora crassa, a filamentous fungus, is inhibited by TTC under certain conditions. If a strain of Neurospora possesses the TTC^S allele of the gene described in this thesis, and does not have any cytoplasmic mutation, it will be inhibited by TTC. Three major wild type genomes have been studied; they are: Oak Ridge-St. Lawrence, Rockefeller-Lindegren, and Emerson.

Through analyses of reciprocal crosses, both inter- and intrastrain, the response to TTC has been determined to be governed by a single gene. This TTC gene is linked to mating type with very little crossing over in the OR-SL strains, and since both a and A are TTC^R in RL, none are detectable there. The Emerson strains did exhibit crossing over in a relatively small sample. In one ordered ascus the TTC gene

showed 1st division segregation, while mating type showed 2nd division segregation, indicating that the TTC locus was between the mating type locus and the centromere, and that there was some distance between them. In all other crosses involving any of the wild types the TTC gene segregated with mating type, whether it was 1st or 2nd division segregation. In the one Emerson ascus then, either the mating type or TTC locus had changed its relative position in linkage group I. Since TTC exhibited 1st division segregation, the mating type locus was assumed to have moved. In subsequent analyses of ordered tetrads from intrastrain crosses done in this laboratory the Emerson mating type locus was found to be at least 4 times farther from the centromere than in the other two wild type strains--OR-SL and RL. This shift in the mating type locus could have been brought about by an inversion in linkage group I and there is some evidence that an inversion of this type does occur. Rifaat (1958), while studying several loci in linkage group I, discovered that in some strains arg-3 is distal to mt with respect to the centromere, while in others the order is reversed. He states that the cause of the reversal is an inversion. If we assume the increased centromere distance of the mt locus in the Emerson strains is due to an inversion which does not include the TTC locus, the effect would be to give approximately 15 map units between mt and the TTC locus. Crossing over within this map distance would be frequent enough to account for the relative ease

with which recombinants were detected in the Emerson crosses. An inversion in only one mating type would necessarily complicate pairing during meiosis resulting in a reduced recombination rate. An interstrain cross of Emerson and OR-SL, which would lack this inversion, did not yield recombinants, thereby strengthening the inversion hypothesis.

That a single gene controls a strain's response to TTC has been determined by the data; however, the same data suggest that there are other factors involved. When the TTC^r allele is present in the OR-SL a it is not as resistant at the various concentrations as an RL a with the same allele. Also the RL A shows more resistance than the OR-SL A, both of which carry the resistant allele. There are several known differences between the OR-SL strains and the RL strains. For example, they carry different alleles for heterocaryon compatibility, and the RL strains are subject to the spontaneous development of a cytoplasmically-determined abnormal phenotype. Therefore, when the TTC^r allele is present in these two strains, it resides in two different genomes and two different plasmons (plasmon is the term denoting all the extrachromosomal hereditary information). The concept of a different plasmon altering response to TTC can be seen in another way. The [mi-1] strains are respiratory-deficient cytoplasmic mutants. Among other changes these mutants have altered mitochondria; hence a different plasmon. When the OR-SL a has the TTC^s allele it cannot tolerate 50 µg/ml TTC;

however, when this same a TTC^s is present in an [mi-1] cytoplasmic mutant it can tolerate that concentration of TTC. This resistance of the [mi-1] strains as a group could not be attributed to the nuclear resistance because the effect was additive when tested.

The spot test technique for tetrad analysis of TTC response which works so well for wild types and nutritional mutants must be used with caution when scoring extrachromosomal mutants of the [mi-1] type. These mutants are usually slow growers with erratic growth patterns for at least the initial 24 hours after inoculation. Since this is the period during which the spot tests must be scored for valid results, these mutants can give inaccurate data. However, spot tests can still be used to detect relative degrees of resistance within one ascus or among the serially isolated progeny of a cross, if scoring is done carefully.

The question of mechanism with respect to TTC response is most interesting when viewed in terms of these cytoplasmic mutants. According to the work of Palmer and Kalina (1968), the tetrazolium salts are reduced along the electron transport chain, each salt coupled to one of the cytochromes. This explanation is satisfactory for wild type strains with a full complement of cytochromes but [mi-1] does not have a complete set (Table 12). Brock (1958) while reporting on yeasts suggested that the ones which were not inhibited by TTC may be using an alternate pathway for energy metabolism, by-passing the respiratory mitochondrial-like particles.

Tissieres, Mitchell, and Haskins (1953) determined that [mi-1] is not dependent on the cytochrome system for terminal oxidation. They based their conclusion on several facts, one of which was that respiration of intact mycelia of young [mi-1] was not affected by cyanide and azide. The reduction site of TTC is beyond the cyanide/azide-sensitive point in the electron transport chain (Palmer and Kalina, 1968). So it is quite possible that the resistance of [mi-1] could simply be due to the lack of reduction site for TTC. There is at least one other possibility. TTC inhibits electron flow from substrate to cytochrome b (Palmer and Kalina, 1968). Two of the cytoplasmic strains [mi-2] and [mi-4] show some inhibition by TTC; both of these strains have wild type amounts of cytochrome b (Figure 8). In contrast the two cytoplasmic strains which lack cytochrome b, [mi-1] and [mi-1?], are not inhibited by TTC (Table 12). The susceptible strains lack only cytochrome a/a₃, while those which are resistant lack both cytochromes a/a₃ and b. Perhaps the resistant strains by-pass the electron transport system completely; the partially inhibited ones use it as far as they can.

Kalina and Palmer (1968) in their discussion of the reduction rate of the tetrazolium salts mention uptake of these compounds into the mitochondria. Presumably then reduction takes place inside the mitochondria, but in the experiments which involved the observation of hyphae in the

presence of TTC, we noted growth of large, needle-like crystals inside the cells (Figure 5). The color of the crystals ranged from pink through magenta to a definite red, the color of the triphenylformazan or reduced TTC. It would seem then that the salt is reduced molecule by molecule in the mitochondria, escapes from the mitochondria, and aggregates to form visible crystals in the cytoplasm. Crystals present usually disappear within 4 hours, suggesting *Neurospora* has the ability to slowly convert the formazan to a soluble compound.

At a given concentration of TTC (2 mg/ml), the a mating type required less time to develop these crystals. This observation agrees with the response of conidia germinating on a TTC-minimal agar cover slip. In that experiment also, the a showed large abnormal vacuoles within 24 hours in the presence of an inhibitory concentration of TTC. The A, in the presence of the much higher inhibitory concentration for it, showed numerous small vacuoles in 20 hours similar to those seen in a in 5 hours. When 10 mg/ml TTC was injected into the hyphae of A and a, there was no difference in response. All of these results:

- 1) earlier crystal formation in a mating type
- 2) A conidia requiring 4 times as long to exhibit the same response as a to an inhibitory concentration of TTC
- 3) no difference in response between A and a when injected with TTC

support the hypothesis that in some way the different alleles of the gene for TTC response alter membrane permeability. The third observation mentioned tentatively eliminates a difference in metabolism between the two mating types since there was no detectable variation in their response to TTC when it was injected under the same conditions into A and a hyphae.

The work of deTerra and Tatum (1961) concerning alteration of the cell wall when *Neurospora* is grown in the presence of sorbose, coupled with the increased inhibition shown when TTC was used in the presence of sorbose, can also be cited as additional evidence for the permeability hypothesis. The weakened and altered cell wall could be a prime target for TTC especially if its ability to inhibit is merely a manifestation of its transport through the cell wall.

The discovery that TTC in suitable concentrations will inhibit wild type but not [mi-1] suggests a possible approach to the problem of finding new cytoplasmic mutants. Presumably, wild-type conidia could be treated with mutagenic agents and then plated on TTC. Wild-type conidia would not develop, but mutants resistant to TTC would do so. Among these mutants there should be some of cytoplasmic origin which could easily be recovered.

With current procedures, about 10 weekly serial transfers are required to produce evidence of successful mitochondrial transplantation. From patterns observed in Figure 6,

it should be possible to sorbose plate strains after one transfer and predict which ones were successful transplants. A system using this technique should be devised and tested for use in future transplant studies.

A more direct approach, possibly incubating isolated mitochondria in the presence of TTC, could determine whether the mitochondria are viable even before transplantation. Such a technique would be extremely valuable in a study of the effect of various treatments (i.e. sonification, repeated freezing and thawing) on the viability of mitochondria.

Several of the tetrazolium salts were incorporated into the medium for sorbose plating various cytoplasmic mutants. These mutants possess different cytochrome complements and hopefully some correlation would be seen between the reduction site of the salt and the status of the mutant with regard to this site in its cytochrome chain. Unfortunately, in the very limited experiment carried out using the same concentration of each salt, no correlation was evident. If time and resources permit, further investigation into this area should be attempted. A definite correlation would provide not only some insight into the cytoplasmic resistance of *Neurospora* to TTC, but also could suggest some possibilities for using the tetrazolium salts as an assay method for mitochondrial recombination.

SUMMARY

The tetrazolium salt, 2,3,5-triphenyltetrazolium chloride (TTC), inhibits germination of conidia of all Neurospora crassa strains tested in concentrations exceeding 600 $\mu\text{g/ml}$. At 50 $\mu\text{g/ml}$ there is a dichotomous response to the salt. Some strains are completely inhibited; others, completely resistant. Tetrad analyses of crosses between resistant and sensitive strains provided evidence for the existence of both nuclear and extrachromosomal determinants of the dichotomous response. The nuclear determinants (TTC^r, TTC^s) are alleles of a single gene, closely linked to mating type in linkage group I. The extrachromosomal determinant is associated with the maternally inherited characteristics of [mi-1], and, to a lesser extent, with those of [mi-2] and [mi-4]. In the St. Lawrence-Oak Ridge strains and the Emerson strains mating type A is TTC^r, mating type a is TTC^s. Both mating types of the Rockefeller-Lindegren strains are TTC^r. An [mi-1] strain with a TTC^r nuclear allele is more resistant than an [mi-1] strain with a TTC^s allele, suggesting a different mechanism for nuclear and cytoplasmic-based resistance.

Experimental results show that the TTC effect is inhibitory, not lethal, and is restricted to the ungerminated conidium. Conidia incubated in the presence of TTC before plating on TTC-free medium were not inhibited, indicating the

effect was reversible. Sorbose present in the incubation medium caused a slight increase in inhibition.

A concentration of TTC inhibitory for mating type a induced abnormal vacuolation in a conidia but had no effect on A. In the presence of TTC, red crystals could be observed in vegetative hyphae of mating type a at least one hour before they were visible in A, but both mating types showed identical response to microinjected TTC. This difference in effect of externally and internally applied TTC suggests that the response of the *Neurospora* mating types is based on a variation in membrane permeability, rather than on metabolism. The mitochondrial mutant [mi-1] is resistant to TTC even when it has the TTC^S nuclear allele, and the [mi-1] phenotype eventually replaces that of wild type when a mixture of the two types of mitochondria are present in a common cytoplasm. Such a mixture (heterochondrion) can be produced by microinjection of [mi-1] mitochondria into wild type. It was then possible to follow the change in mitochondrial populations in the heterochondrion by plating conidia on medium containing TTC.

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